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(71) Applicant *(for all designated States except US)*: DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SIBBESEN, Ole [DK/DK]; Værebrovej 117B, DK-2880 Bagsværd (DK). SØRENSEN, Jens, Frisbæk [DK/DK]; Nordvestpassagen 93, DK-8200 Aarhus (DK).

(74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co, 21 New Fetter Lane, London EC4A 1DA (GB).

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(57) Abstract: The present invention relates to a variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor as compared with the parent enzyme.

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ENZYME

Field of the invention

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The present invention relates to mutant xylanase enzymes having an altered sensitivity to xylanase inhibitors. The present invention also relates to the use of these mutant enzymes in processing plant materials.

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Background to the invention

For many years, endo-β-1,4-xylanases (EC 3.2.1.8) (referred to herein as xylanases) have been used for the modification of complex carbohydrates derived from plant cell wall material. It is well known in the art that the functionality of different xylanases (derived 15 from different micro organisms or plants) differs enormously.

Comprehensive studies characterising the functionality of xylanases have been done on well characterised and pure substrates (Kormelink *et al.*, 1992). These studies show that different xylanases have different specific requirements with respect to substitution of the 20 xylose backbone of the arabinoxylan (AX). Some xylanases require three un-substituted xylose residues to hydrolyse the xylose backbone; others require only one or two. The reasons for these differences in specificity is thought to be due to the three dimensional structure within the catalytic domains, which in turn is dependent on the primary structure of the xylanase, i.e. the amino acid sequence. However, the translation of these 25 differences in the amino acid sequences into differences in the functionality of the xylanases, has up until now not been documented when the xylanase acts in a complex environment, such as plant material.

The xylanase substrates found in wheat (wheat flour), have traditionally been divided into 30 two fractions: The water un-extractable AX (WU-AX) and the water extractable AX (WE-AX). The WU-AX:WE-AX ratio is approx. 70:30 in wheat flour. There have been numerous explanations as to why there are two different fractions of AX. The older literature (D'Appolonia and MacArthur (1976) and Montgomery and Smith (1955))

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describes quite high differences in the substitution degree between WE-AX and WU-AX. The highest degree of substitution was found in WE-AX. This was used to explain why some of the AX was extractable. The high degree of substitution made the polymer soluble, compared to a lower substitution degree, which would cause hydrogen bonding between polymers and consequently precipitation.

The difference between the functionality of different xylanases has been thought to be due to differences in xylanase specificity and thereby their preference for the WU-AX or the WE-AX substrates.

In some applications (e.g. bakery) it is desirable to produce high molecular weight (HMW) soluble polymers from the WU-AX fraction. Such polymers have been correlated to a volume increase in bread making (Rouau, 1993; Rouau *et al.*, 1994 and Courtin *et al.*, 1999).

In other applications it is desirable to modify the HMW WU-AX, making the molecular weight lower, reducing their hydrocolloid effect and hence water-binding in the product (crackers, flour separation, etc.)

These different applications require different functionalities of the xylanases used to do the job. As mentioned above, the difference in functionality has been explained by the different substrate specificities of the xylanases.

Summary of the invention

By contrast to earlier studies, we have now shown that other factors are more important in determining xylanase functionality than the substrate specificity of the xylanases determined on pure well-characterised substrates. The data presented herein show that endogenous xylanase inhibitors dictate the functionality of the xylanases currently used in, for example, wheat flour systems. This means that a xylanase that normally modifies the WU-AX, giving increased dough liquid viscosity in a wheat flour system, has a different functionality if the endogenous xylanase inhibitor is absent in the wheat flour. Thus, our

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findings indicate that the design and application of uninhibited xylanases, for example, using site-directed mutagenesis could be a way to mimic the absence of xylanase inhibitors in various plant materials, giving new xylanases with completely new functionality. Such xylanases would be very effective in applications where a reduction in viscosity is required. The uninhibited xylanase would act rapidly on the AX, and be primarily influenced by its specific activity, rather than by endogenous inhibitors. From our studies, we consider that the inhibitory effects are likely to be far more important than the specific activity. Indeed our results show for the first time that there are 10 to 50 fold differences in inhibition levels between the family 11 xylanases.

Furthermore, we have gone on to design and test a series of xylanases modified by site-directed mutagenesis to demonstrate that xylanases can be produced that have reduced sensitivity to xylanase inhibitors present in plant materials. In particular, we have identified a number of residues in family 11 xylanases which influence the degree of inhibition of the xylanase.

Thus, it will be possible to produce variant xylanases having reduced sensitivity to xylanase inhibitors and hence altered functionality. This will, for example, allow a reduction in the amount of xylanase required in a number of applications such as animal feed, starch production, bakery, flour separation (wetmilling) and, paper and pulp production.

Accordingly, the present invention provides a variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor as compared with the parent enzyme.

Here, the "parent enzyme" is the xylanase enzyme from which the variant xylanase enzyme is derived or derivable. With respect to the term "derivable", the variant need not necessarily be derived from the parent enzyme. Instead, the variant could be prepared, for example, by use of recombinant DNA techniques that utilise nucleotide sequence(s) encoding said variant xylanase sequence - i.e. here the nucleotide sequence(s) are similar

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to mutated nucleotide sequence(s) but they are not prepared by mutation of the parent nucleotide sequence(s). The variant may even be prepared by chemically modifying a parent enzyme.

5 For some embodiments the parent enzyme is the wild type enzyme. The term "wild type" is a term of art understood by skilled persons and includes a phenotype that is characteristic of most of the members of a species occurring naturally and contrasting with the phenotype of a mutant. Thus, in the present context, the wild type enzyme may be a form of the enzyme naturally found in most members of the relevant species. 10 Generally, the relevant wild type enzyme in relation to the variant polypeptides of the invention is the most closely related corresponding wild type enzyme in terms of sequence homology. For example, for the particular mutant xylanases described in the examples, the corresponding wild type enzyme is the wild type *B. subtilis* xylanase A, more specifically the wild type *B. subtilis* xylanase A published by Paice *et al*., 1986 and shown 15 as SEQ I.D. 1. However, where a particular wild type sequence has been used as the basis for producing a variant polypeptide of the invention, this will be the corresponding wild type sequence regardless of the existence of another wild type sequence that is more closely related in terms of amino acid sequence homology.

20 For some embodiments, preferably the variant polypeptide is derived from a family 11 xylanase.

One of our surprising findings is that in our studies so far a mutation in the xylanase active site has no measurable effect on inhibition against the xylanase inhibitor. This is in 25 direct contrast to the mutation(s) that are made outside of the active site – which mutations are discussed in more detail below.

In a preferred aspect the amino acid modification is of one or more surface amino acid residues.

In a more preferred aspect the amino acid modification is of one or more solvent accessible residues. Here, the solvent is water.

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In a more preferred aspect the amino acid modification is of one or more surface residues outside of the active site.

In a highly preferred aspect the amino acid modification is of one or more surface residues
5 outside of the active site and which is/are at least 8 % solvent accessible. Here, the solvent is water.

In a highly preferred aspect the amino acid modification is of one or more surface residues outside of the active site and which is/are at least 10 % solvent accessible. Here, the solvent is water.

10

Solvent accessibility can be determined using Swiss-PdbViewer (version 3.5b1), which can be located via the internet at http://www.expasy.ch/spdbv/mainpage.html. The Swiss-PdbViewer is presented by Glaxo Wellcome Experimental Research.

15 The surface amino acids of xylanase enzymes are determinable by a person skilled in the art.

By way of example, the *B. subtilis* amino acid sequence for xylanase A is shown as SEQ I.D. No. 1. With respect to this sequence, the surface amino acid residues are:

20

Ala1-Trp6, Asn8, Thr10-Gly23, Asn25, Ser27, Asn29, Ser31-Asn32, Gly34, Thr43-Thr44, Ser46-Thr50, Asn52, Asn54, Gly56-Asn61, Asn63, Arg73-Leu76, Thr87-Arg89, Thr91-Lys95, Thr97, Lys99, Asp101-Gly102, Thr104, Thr109-Thr111, Tyr113-Asn114, Asp119-Thr124, Thr126, Gln133-Asn141, Thr143,
25 Thr145, Thr147-Asn148, Asn151, Lys154-Gly157, Asn159-Leu160, Ser162-Trp164, Gln175, Ser177, Ser179, Asn181, Thr183, Trp185.

As indicated, the surface amino acids of other xylanase enzymes (such as *Thermomyces lanuginosus* xylanase A, whose coding nucleotide sequence is presented as SEQ ID No.
30 9) are determinable by a person skilled in the art.

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Hence, for some aspects the present invention encompasses a variant xylanase polypeptide, or fragment thereof having xylanase activity, which variant xylanase polypeptide or fragment comprises one or more amino acid modifications at any one of amino acid residues:

Ala1-Trp6, Asn8, Thr10-Gly23, Asn25, Ser27, Asn29, Ser31-Asn32, Gly34, Thr43-Thr44, Ser46-Thr50, Asn52, Asn54, Gly56-Asn61, Asn63, Arg73-Leu76, Thr87-Arg89, Thr91-Lys95, Thr97, Lys99, Asp101-Gly102, Thr104, Thr109-Thr111, Tyr113-Asn114, Asp119-Thr124, Thr126, Gln133-Asn141, Thr143, Thr145, Thr147-Asn148, Asn151, Lys154-Gly157, Asn159-Leu160, Ser162-Trp164, Gln175, Ser177, Ser179, Asn181, Thr183, Trp185

of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or its/their equivalent positions in other homologous xylanase polypeptides.

Thus, in one embodiment, the present invention provides a variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications at any one of amino acid residues numbers:

11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 and 175

of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

In one embodiment, the present invention provides a variant xylanase polypeptide or fragment thereof having xylanase activity, comprising one or more amino acid modifications at any one of amino acid residues numbers 11, 12 and 13 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

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Specific preferred examples of modifications made are presented in the Examples section herein.

For some embodiments, preferably the variant xylanase polypeptide, or fragment thereof having xylanase activity, comprises one or more amino acid modifications at any one of amino acid residues numbers: 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 29, 30, 31, 32, 33, 34, 35, 36, 37, 61, 62, 63, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 173, 174, 175, 176, 177, 178 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

For convenience, we sometimes refer to the amino acid residues numbers: 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 29, 30, 31, 32, 33, 34, 35, 36, 37, 61, 62, 63, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 173, 174, 175, 176, 177, 178 as BAND 1.

Figure 1 shows the 3-D structure of *B. subtilis* xylanase having the amino acid sequence shown as SEQ I.D. No. 1. BAND 1 is depicted in Figure 1 as the upper layer of the molecule and extends approximately 13 Ångstroms from the top of the molecule when the molecule is orientated as shown in Figure 1. BAND 1 ends with the residue Phe 125 on the left hand side when viewing Figure 1 and with the residue Asn 61 on the right hand side when viewing Figure 1.

In addition, or in the alternative, for some embodiments, preferably the variant xylanase polypeptide, or fragment thereof having xylanase activity, comprises one or more amino acid modifications at any one of the other amino acid residues.

Preferably said other modifications may occur at any one or more of amino acid residues numbers: 3, 4, 5, 6, 7, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 38, 39, 40, 41, 42, 43, 44, 45, 55, 56, 57, 58, 59, 60, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 108, 109, 110, 126, 127, 128, 129, 130, 131, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 179, 180,

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181, 182, 183 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

For convenience, we sometimes refer to the amino acid residues numbers: 3, 4, 5, 6, 7, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 38, 39, 40, 41, 42, 43, 44, 45, 55, 56, 57, 58, 59, 60, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 108, 109, 110, 126, 127, 128, 129, 130, 131, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 179, 180, 181, 182, 183 of the *B. subtilis* amino acid sequence shown as BAND 2.

Preferably said other modifications may occur at any one or more of the surface amino acid residues numbers: 3, 4, 5, 6, 19, 20, 21, 22, 23, 25, 27, 43, 44, 56, 57, 58, 59, 60, 73, 74, 75, 76, 87, 89, 91, 92, 93, 94, 109, 110, 126, 159, 160, 162, 163, 164, 179, 181, 183 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

Preferably, the present invention encompasses a variant xylanase polypeptide, or fragment thereof having xylanase activity, which comprises one or more amino acid modifications in BAND 1 and optionally/or BAND 2 of the *B. subtilis* amino acid sequence or their equivalent positions (bands) in other homologous xylanase polypeptides. Hence, the modification is in at least BAND 1; but could be in just BAND 2 alone.

The variant xylanase polypeptide may comprise other modifications in other amino acid residues, such as modification at any one of amino acid residues: 1, 2, 46, 47, 48, 49, 50, 51, 52, 53, 54, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 184, 185 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

The variant xylanase polypeptide may comprise other modifications in other surface amino acid residues, such as modification at any one of the surface amino acid residues: 1, 2, 46, 47, 48, 49, 50, 52, 54, 95, 97, 99, 101, 102, 104, 133, 134, 135, 136, 137, 138,

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139, 140, 141, 143, 145, 147, 148, 151, 154, 155, 156, 157, 185 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

5 Preferably, the inhibitor is an inhibitor found naturally in plant tissues. Preferably the sensitivity of the variant xylanase enzyme to the inhibitor is reduced as compared to the parent xylanase enzyme.

The present invention also provides a nucleic acid molecule (a nucleotide sequence) 10 encoding a polypeptide of the invention. Also provided is a vector comprising a nucleic acid of the invention, optionally operably linked to a regulatory sequence capable of directing expression of said nucleic acid in a suitable host cell. A host cell comprising a nucleic acid or a vector of the invention is also provided.

15 In another aspect the present invention provides a method of making a polypeptide of the invention comprising transforming a host cell with a nucleic acid encoding said polypeptide, culturing the transformed cell and expressing said polypeptide.

Our results show that these variant polypeptides have improved properties that make them 20 suitable for a variety of applications, such as bakery, animal feed, starch production, flour separation (wetmilling) and, paper and pulp production.

Accordingly, the present invention also provides the use of a variant polypeptide of the invention in a method of modifying plant materials.

25

Also provided is the use of a variant polypeptide of the invention in baking. The invention further provides the use of a variant polypeptide of the invention in processing cereals, starch production and animal feed and the use of a variant polypeptide of the invention in processing wood, for example in enhancing the bleaching of wood pulp.

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In a further aspect, the present invention provides a method of altering the sensitivity of a xylanase polypeptide to an inhibitor which method comprises modifying one or more

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amino acid residues of said enzyme selected from amino acid numbers 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 and 175 based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides. Preferably the sensitivity is reduced.

5

Importantly, our results also show for the first time that xylanase inhibitors play an important role in determining the functionality of xylanase enzymes in a complex system, such as a plant material. By the term "functionality", we mean the biochemical properties of the xylanase in a given system. These properties include substrate specificity, K_m and
10 V_{max} kinetic parameters (where appropriate) and the nature of the reaction products obtained by the action of the xylanase in that system. Functionality may also consequently be described in terms of the effect on the physical and/or chemical properties of the plant materials on which the xylanase acts, for example the extent to which the viscosity of the material is altered.

15

In the same way that variant xylanases may be used in a variety of processing applications, xylanase inhibitors may be used in a variety of processing applications such as bakery, wood pulp processing and cereal processing.

20 Detailed description of the invention

Although in general any molecular techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed,
25 John Wiley & Sons, Inc.

A. Variant xylanase polypeptides

Xylanase enzymes have been reported from nearly 100 different organisms, including
30 plants, fungi and bacteria. The xylanase enzymes are classified into several of the more than 40 families of glycosyl hydrolase enzymes. The glycosyl hydrolase enzymes, which include xylanases, mannanases, amylases, β-glucanases, cellulases, and other

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carbohydrases, are classified based on such properties as the sequence of amino acids, the three dimensional structure and the geometry of the catalytic site (Gilkes, et al., 1991, Microbiol. Reviews 55: 303-315).

5 Of particular interest for baking applications are the enzymes classified in Family 11. All of these are xylanases and are known as the "Family 11 xylanases". Some publications refer to these synonymously as the Family G xylanases, but the term "Family 11 xylanases" will be used herein to refer to both Family G and Family 11 xylanases.

10 Table A lists a number of known Family 11 xylanases. Most of them have a molecular mass of about 21,000 Da. Three of the Family 11 xylanases (*Clostridium stercorarium* XynA, *Streptomyces lividans* XynB, and *Thermomonospora fusca* XynA) have a higher molecular mass of 31,000 to 50,000 Da. However, these xylanases have a catalytic core sequence of about 21,000 Da similar to the other Family 11 xylanases. The amino acid

15 sequences of the Family 11 xylanases (or, for the larger enzymes, the catalytic core) show a high degree of similarity, usually with more than 40 % identical amino acids in a proper amino acid alignment. The Family 11 xylanases, which are of bacterial, yeast, or fungal origin, share the same general molecular structure.

20 Figure 2 shows amino acid sequence alignment data in respect of 51 Family 11 xylanases.

TABLE A - Family 11 xylanases

Aspergillus niger Xyn A	*Aspergillus kawachii* Xyn C
Aspergillus tubigensis Xyn A	*Bacillus circulans* Xyn A
Bacillus pumilus Xyn A	*Bacillus subtilis* Xyn A
Cellulomonas fimi Xyn D	*Chainia* spp. Xyn
Clostridium acetobutylicum Xyn B	*Clostridium stercorarium* Xyn A
Fibrobacter succinogenes Xyn C	*Neocallimastix patriciarum* Xyn A
Nocardiopsis dassonvillei Xyn II	*Ruminococcus flavefaciens* Xyn A

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Schizophyllum commune Xyn	*Streptomyces lividans* Xyn B
Streptomyces lividans Xyn C	*Streptomyces sp.* No. 36a Xyn
Streptomyces thermoviolaceus Xyn II	*Thermomonospora fusca* Xyn A
Trichoderma harzianum Xyn	*Trichoderma reesei* Xyn I
Trichoderma reesei Xyn II	*Trichoderma viride* Xyn

Variant xylanases of the invention

A variant xylanase polypeptide of the invention is typically obtained by modifying a
5 xylanase polypeptide by substituting, deleting or adding one or more amino acid residues within the amino acid sequence of the xylanase polypeptide. Preferably the modification comprises one or more amino acid substitutions. Modification of polypeptide sequences can be carried out using standard techniques such as site directed mutagenesis. The modification may also occur by chemical techniques – such as chemical modification of
10 one or more amino acid residues.

The starting sequence may be a wild type sequence or a non-naturally occurring sequence, for example a derivative that has already been subjected to protein engineering. The xylanase sequence to be modified may be from any source, for example a bacterial, fungal
15 or plant source. Preferably the xylanase sequence to be modified is that of a Family 11 xylanase, more preferably a Family 11 xylanase selected from *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma harzianum* xylanase, *Trichoderma viride* xylanase, *Bacillus circulans* xylanase A, *Bacillus subtilis* xylanase A, *Aspergillus niger* xylanase A, *Aspergillus kawachii xylanase* C, *Aspergillus tubigensis*
20 xylanase A, *Streptomyces lividans* xylanase B, and *Streptomyces lividans* xylanase C.

In a particularly preferred embodiment, the xylanase sequence to be modified is the *B subtilis* xylanase sequence shown as SEQ ID No. 1 or a homologue thereof. Preferably said homologue has at least 40, 50, 60 or 80% homology over at least 50 or 100 amino
25 acid residues as determined using the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387).

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Specific modifications that are preferred according to the present invention include one or more amino acid substitutions at positions 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 and 175 based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

Particularly preferred substitutions include one or more of D11şY, D11şN, D11şF, D11şK, D11şS, D11şW, G12şF, G13şF, I15şK, N17şK, N17şY, N17şD, N29şK, N29şY, N29şD, S31şK, S31şY, S31şD, N32şK, G34şD, G34şF, G34şT, Y113şA, Y113şD, Y113şK, N114şA, N114şD, N114şF, N114şK, D119şK, D119şY, D119şN, G120şK, G120şD, G120şF, G120şY, G120şN, D121şN, D121şK, D121şF, D121şA, R122şD, R122şF, R122şA, T123şK, T123şY, T123şD, T124şK, T124şY, T124şD, Q175şE, Q175şS and Q175şL (with reference to the amino acid sequence of *B. subtilis* xylanase) or their equivalents in other homologous xylanase polypeptides. Further references to specific residues of the *B. subtilis* xylanase shown as SEQ ID No. 1 will also include their equivalents in other homologous xylanase polypeptides.

A combination of mutations may be carried out, for example mutations at two or more of the above-mentioned residues. Examples of such combinations are presented in the Examples section herein.

In a further embodiment, the variant polypeptides of the invention may be purified and isolated naturally occurring mutant xylanases. Alternatively, mutant xylanases may be generated by subjecting organisms to mutagens and then screening for individuals comprising mutations in their xylanase genes. Naturally occurring mutants and mutants generated by random mutagenesis may be identified/screened using a variety of techniques such as PCR screening using suitable nucleic acid primers to amplify regions of xylanase genes and sequencing the resulting fragments.

Thus variant polypeptides of the invention include naturally occurring mutant xylanases (purified and isolated from the organisms in which they occur or obtained recombinantly),

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mutant xylanases obtained by random mutagenesis and mutant xylanases obtained by site-directed mutagenesis.

Variant polypeptides of the invention may also be subjected to further modifications that do not necessarily affect sensitivity to inhibitors, including any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant amino acid sequence retains xylanase activity, preferably having at least substantially the same xylanase activity as the unmodified sequence.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention also include fragments of the full length sequences mentioned above having xylanase activity.

Polypeptides of the invention may further comprise heterologous amino acid sequences, typically at the N-terminus or C-terminus, preferably the N-terminus. Heterologous sequence may include sequences that affect intra or extracellular protein targeting (such as leader sequences).

Polypeptides of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques

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well known to skilled persons such as solid phase synthesis. Polypeptides of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences, such as a thrombin cleavage site. Preferably the fusion protein will not hinder the function of the protein sequence of interest.

The use of appropriate host cells is expected to provide for such post-translational modifications as may be needed to confer optimal biological activity on recombinant expression products of the invention.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a polypeptide of the invention.

Variant polypeptides of the invention have altered sensitivity to xylanase inhibitors compared to the parent xylanase sequence – which may be a corresponding wild type xylanase. Preferably, variant polypeptides have reduced sensitivity to xylanase inhibitors. The term "altered sensitivity to xylanase inhibitors" means that extent to which the endo-β-1,4-xylanase activity of a variant polypeptide of the invention is inhibited by the xylanase inhibitor is different to that of the parent xylanase enzyme – which may be the corresponding wild type xylanase. Preferably the extent to which the variant polypeptide is inhibited by the inhibitor is less than that of the parent xylanase enzyme – which may be the wild type protein. This may, for example, be due to a change in the three-dimensional structure of the variant polypeptide such that the inhibitor no longer binds with the same affinity as it does to the parent xylanase enzyme – which may be the wild type enzyme.

The sensitivity of the variant polypeptides of the invention to xylanase inhibitors can be assayed using, for example, the assay described in example 4 and below. A suitable

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inhibitor for use in the assay is the inhibitor purified from wheat flour in example 1. Other inhibitors are described below.

Xylanase assay (Endo-β-1,4-Xylanase activity)

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Xylanase samples are diluted in citric acid (0.1 M) - *di*-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0, to obtain approx. OD = 0.7 in the final assay. Three dilutions of the sample and an internal standard with a defined activity are thermostated for 5 minutes at 40°C. At time = 5 minutes, 1 Xylazyme tab (crosslinked, dyed xylan substrate) is
10 added to the enzyme solution. At time = 15 minutes (or in some cases longer, depending on the xylanase activity present in the sample) the reaction is terminated, by adding 10 ml of 2% TRIS. The reaction mixture is centrifuged and the OD of the supernatant is measured at 590 nm. Taking into account the dilutions and the amount of xylanase, the activity (TXU, Total-Xylanase-Units) of the sample can be calculated relative to the
15 standard.

Xylanase inhibitors

As used herein, the term "xylanase inhibitor" refers to a compound, typically a protein,
20 whose role is to control the depolymerisation of complex carbohydrates, such as arabinoxylan, found in plant cell walls. These xylanase inhibitors are capable of reducing the activity of naturally occurring xylanase enzymes as well as those of fungal or bacterial origin. Although the presence of xylanase inhibitors have been reported in cereal seeds (see for example McLauchlan et al 1999a; Rouau and Suget 1998) their impact on the
25 efficacy of xylanase enzymes has not been extensively examined.

McLauchlan et al (1999a) disclose the isolation and characterisation of a protein from wheat that binds to and inhibits two family-11 xylanases. Likewise, WO 98/49278 demonstrates the effect of a wheat flour extract on the activity of a group of microbial
30 xylanases all of which are classified as family 11 xylanases. Debyser et al. (1999) also disclose that endoxylanases from *Aspergillus niger* and *Bacillus subtilis*, which are both members of the family 11 xylanases were inhibited by a wheat xylanase inhibitor called

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TAXI. McLauchlan *et al* (1999b) teach that extracts from commercial flours such as wheat, barley, rye and maize are capable of inhibiting both family 10 and 11 xylanases.

The xylanase inhibitor may be any suitable xylanase inhibitor. By way of example, the
5 xylanase inhibitor may be the inhibitor described in WO-A-98/49278 and/or the xylanase inhibitor described by Rouau, X. and Surget, A. (1998), McLauchlan, R., et al. (1999) and/or the xylanase inhibitor described in UK patent application number 9828599.2 (filed 23 December 1998), UK patent application number 9907805.7 (filed 6 April 1999) and UK patent application number 9908645.6 (filed 15 April 1999).

10

Xylanase inhibitor assay

100 µl of an candidate inhibitor fraction, 250 µl xylanase solution (containing 12 TXU microbial xylanase/ml) and 650 µl buffer (0.1 M citric acid - 0.2M *di*-sodium hydrogen
15 phosphate buffer, pH 5.0) are mixed. The mixture is thermostated for 5 minutes at 40.0°C. At time = 5 minutes one Xylazyme tab is added. At time = 15 minutes the reaction is terminated by adding 10 ml 2% TRIS. The reaction mixture is centrifuged (3500 g, 10 minutes, room temperature) and the supernatant is measured at 590 nm. The inhibition is calculated as residual activity compared to the blank. The blank is prepared
20 the same way, except that the 100 µl inhibitor is substituted with 100 µl buffer (0.1 M citric acid - 0.2 M *di*-sodium hydrogen phosphate buffer, pH 5.0).

Specific xylanase inhibitor

25 As indicated, a xylanase inhibitor that may be used in accordance with the present invention is the xylanase inhibitor described in UK patent application number 9828599.2 (filed 23 December 1998), UK patent application number 9907805.7 (filed 6 April 1999) and UK patent application number 9908645.6 (filed 15 April 1999).

30 This endogenous endo-β-1,4-xylanase inhibitor is obtainable from wheat flour. The inhibitor is a di-peptide, having a MW of about 40 kDa (as measured by SDS-PAGE or mass spectrometry) and a pI of about 8 to about 9.5.

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Sequence analysis to date has revealed the that the inhibitor has at least one or more of the sequences presented as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7 and/or SEQ ID No. 8.

5

These inhibitors described in the prior art may also be used in assays to determine the sensitivity of a variant polypeptide of the invention to xylanase inhibitors. They may also be used as described below to modulate the functionality of a xylanase.

10 Polynucleotides

Polynucleotides of the invention comprise nucleic acid sequences encoding the variant polypeptide sequences of the invention. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the
15 degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any particular host organism in which the polypeptides of the invention are to be expressed.

20 Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5'
25 ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

30 Nucleotide vectors and host cells

Polynucleotides of the invention can be incorporated into a recombinant replicable vector.

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The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under
5 conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast and fungi.

Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence by the
10 host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. The term "regulatory sequences"
15 includes promoters and enhancers and other expression regulation signals.

Enhanced expression of the polynucleotide encoding the polypeptide of the invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions, which serve to increase expression and, if desired,
20 secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the invention.

Aside from the promoter native to the gene encoding the polypeptide of the invention, other promoters may be used to direct expression of the polypeptide of the invention. The
25 promoter may be selected for its efficiency in directing the expression of the polypeptide of the invention in the desired expression host.

In another embodiment, a constitutive promoter may be selected to direct the expression of the desired polypeptide of the invention. Examples of strong constitutive and/or
30 inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (*xln*A), phytase, ATP-synthetase, subunit 9 (*oli*C), triose phosphate isomerase (*tpi*), alcohol dehydrogenase (*Adh*A),

α-amylase (*amy*), amyloglucosidase (AG - from the *gla*A gene), acetamidase (*amd*S) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters.

Examples of strong yeast promoters are those obtainable from the genes for alcohol
5 dehydrogenase, lactase, 3-phosphoglycerate kinase and triosephosphate isomerase.

Examples of strong bacterial promoters are the α-amylase and *SP02* promoters as well as promoters from extracellular protease genes.

10 Hybrid promoters may also be used to improve inducible regulation of the expression construct.

Often, it is desirable for the polypeptide of the invention to be secreted from the expression host into the culture medium from where the polypeptide of the invention may
15 be more easily recovered. According to the present invention, the polypeptide of the invention's native secretion leader sequence may be used to effect the secretion of the expressed polypeptide of the invention. However, an increase in the expression of the polypeptide of the invention sometimes results in the production of the protein in levels beyond that which the expression host is capable of processing and secreting, creating a
20 bottleneck such that the protein product accumulates within the cell. Accordingly, the present invention also provides heterologous leader sequences to provide for the most efficient secretion of the polypeptide of the invention from the chosen expression host.

According to the present invention, the secretion leader may be selected on the basis of
25 the desired expression host. A heterologous secretion leader may be chosen which is homologous to the other regulatory regions of the expression construct. For example, the leader of the highly secreted amyloglucosidase (AG) protein may be used in combination with the amyloglucosidase (AG) promoter itself, as well as in combination with other promoters. Hybrid signal sequences may also be used with the context of the present
30 invention.

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Examples of preferred heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*gla*A - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the α-factor gene (yeasts e.g. *Saccharomyces* and *Kluyveromyces*) or the α-amylase gene (*Bacillus*).

Such vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide of the invention. Thus, in a further aspect the invention provides a process for preparing polypeptides according to the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides. Suitable host cells include, for example, fungal cells, such as *Aspergillus* and yeast cells, such as yeast cells of the genus *Kluyveromyces* or *Saccharomyces*. Other suitable host cells are discussed below.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes. The most suitable selection systems for industrial micro-organisms are those formed by the group of selection markers which do not require a mutation in the host organism. Examples of fungal selection markers are the genes for acetamidase (*amd*S), ATP synthetase, subunit 9 (*oli*C), orotidine-5'-phosphate-decarboxylase (*pvr*A), phleomycin and benomyl resistance (benA). Examples of non-fungal selection markers are the bacterial G418 resistance gene (this may also be used in yeast, but not in fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli uid*A gene, coding for β-glucuronidase (GUS). Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

A further embodiment of the invention provides host cells transformed or transfected with a polynucleotide of the invention. Preferably said polynucleotide is carried in a vector for the replication and expression of said polynucleotides. The cells will be chosen to be

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compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells.

Bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*.

Depending on the nature of the polynucleotide encoding the polypeptide of the invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a fungal host organism should be selected.

A heterologous host may also be chosen wherein the polypeptide of the invention is produced in a form which is substantially free from other xylanases. This may be achieved by choosing a host which does not normally produce such enzymes.

Examples of preferred expression hosts within the scope of the present invention are fungi such as *Aspergillus* species and *Trichoderma* species; bacteria such as *Bacillus* species, *Streptomyces* species and *Pseudomonas* species; and yeasts such as *Kluyveromyces* species and *Saccharomyces* species.

Particularly preferred expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger var. tubigenis*, *Aspergillus niger var. awamori*, *Aspergillus aculeatis*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Trichoderma reesei*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae*.

According to the present invention, the production of the polypeptide of the invention can be effected by the culturing of microbial expression hosts, which have been transformed

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with one or more polynucleotides of the present invention, in a conventional nutrient fermentation medium.

The fermentation medium can comprise a known culture medium containing a carbon source (e.g. glucose, maltose, molasses, etc.), a nitrogen source (e.g. ammonium sulphate, ammonium nitrate, ammonium chloride, etc.), an organic nitrogen source (e.g. yeast extract, malt extract, peptone, etc.) and inorganic nutrient sources (e.g. phosphate, magnesium, potassium, zinc, iron, etc.). Optionally, an inducer may be added.

The selection of the appropriate medium may be based on the choice of expression hosts and/or based on the regulatory requirements of the expression construct. Such media are well-known to those skilled in the art. The medium may, if desired, contain additional components favouring the transformed expression hosts over other potentially contaminating microorganisms.

After fermentation, the cells can be removed from the fermentation broth by means of centrifugation or filtration. After removal of the cells, the variant polypeptide of the invention may then be recovered and, if desired, purified and isolated by conventional means.

Organisms

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the variant xylanase protein according to the present invention and/or products obtained therefrom, wherein a transcriptional regulatory sequence can allow expression of the nucleotide sequence according to the present invention when present in the organism. Suitable organisms may include a prokaryote, fungus, yeast or a plant. For the xylanase aspect of the present invention, a preferable organism may be a bacterium, preferably of the genus Bacillus, more preferably *Bacillus subtilis*.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the protein according to the present

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invention and/or products obtained therefrom, wherein the transcriptional regulatory sequence can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

5

The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

10 Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the amino acid sequence according to the present invention, constructs according to the present invention (including combinations thereof), vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues
15 according to the present invention or the products thereof. The transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from, the cell or organism.

Transformation of Host Cells/Host Organisms

20

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring
25 Harbor Laboratory Press) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999), 4th Ed., John Wiley & Sons, Inc.

If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

30

As mentioned above, a preferred host organism is of the genus Bacillus, such as *Bacillus subtilis*.

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In another embodiment the transgenic organism can be a yeast. In this regard, yeasts have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goodey et al (1987, Yeast Biotechnology, D R Berry et al, eds, pp 401-429, Allen and Unwin, London) and by King et al (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in Saccharomyces cerevisiae and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1

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promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen et al (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H et al (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, e.g. G418.

Another host organism is a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

A transgenic plant of the invention may be produced from any plant such as the seed-bearing plants (angiosperms), and conifers. Angiosperms include dicotyledons and monocotyledons. Examples of dicotyledonous plants include tobacco, (*Nicotiana plumbaginifolia* and *Nicotiana tabacum*), arabidopsis (*Arabidopsis thaliana*), *Brassica napus*, *Brassica nigra*, *Datura innoxia*, *Vicia narbonensis*, *Vicia faba*, pea (*Pisum sativum*), cauliflower, carnation and lentil (*Lens culinaris*). Examples of monocotyledonous plants include cereals such as wheat, barley, oats and maize.

Techniques for producing transgenic plants are well known in the art. Typically, either whole plants, cells or protoplasts may be transformed with a suitable nucleic acid construct encoding a zinc finger molecule or target DNA (see above for examples of nucleic acid constructs). There are many methods for introducing transforming DNA constructs into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods include *Agrobacterium* infection (see, among others, Turpen *et al.*, 1993, J.

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Virol. Methods, 42: 227-239) or direct delivery of DNA such as, for example, by PEG-mediated transformation, by electroporation or by acceleration of DNA coated particles. Acceleration methods are generally preferred and include, for example, microprojectile bombardment. A typical protocol for producing transgenic plants (in particular monocotyledons), taken from U.S. Patent No. 5, 874, 265, is described below.

An example of a method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, non-biological particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming both dicotyledons and monocotyledons, is that neither the isolation of protoplasts nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the tungsten-DNA particles so that they are not delivered to the recipient cells in large aggregates. It is believed that without a screen intervening between the projectile apparatus and the cells to be bombarded, the projectiles aggregate and may be too large for attaining a high frequency of transformation. This may be due to damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more clusters of cells transiently expressing a marker gene ("foci") on the bombarded filter. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 2 to 3.

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After effecting delivery of exogenous DNA to recipient cells by any of the methods discussed above, a preferred step is to identify the transformed cells for further culturing and plant regeneration. This step may include assaying cultures directly for a screenable
5 trait or by exposing the bombarded cultures to a selective agent or agents.

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage, incubating the cells
10 at, e.g., 18°C and greater than 180 μE m^{-2} s^{-1}, and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media.

An exemplary embodiment of methods for identifying transformed cells involves
15 exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

20 To use the bar-bialaphos selective system, bombarded cells on filters are resuspended in nonselective liquid medium, cultured (e.g. for one to two weeks) and transferred to filters overlaying solid medium containing from 1-3 mg/l bialaphos. While ranges of 1-3 mg/l will typically be preferred, it is proposed that ranges of 0.1-50 mg/l will find utility in the practice of the invention. The type of filter for use in bombardment is not believed to be
25 particularly crucial, and can comprise any solid, porous, inert support.

Cells that survive the exposure to the selective agent may be cultured in media that supports regeneration of plants. Tissue is maintained on a basic media with hormones for about 2-4 weeks, then transferred to media with no hormones. After 2-4 weeks, shoot
30 development will signal the time to transfer to another media.

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Regeneration typically requires a progression of media whose composition has been modified to provide the appropriate nutrients and hormonal signals during sequential developmental stages from the transformed callus to the more mature plant. Developing plantlets are transferred to soil, and hardened, e.g., in an environmentally controlled
5 chamber at about 85% relative humidity, 600 ppm CO_2, and 250 $\mu E\ m^{-2}\ s^{-1}$ of light. Plants are preferably matured either in a growth chamber or greenhouse. Regeneration will typically take about 3-12 weeks. During regeneration, cells are grown on solid media in tissue culture vessels. An illustrative embodiment of such a vessel is a petri dish. Regenerating plants are preferably grown at about 19°C to 28°C. After the regenerating
10 plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Genomic DNA may be isolated from callus cell lines and plants to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art
15 such as PCR and/or Southern blotting.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found
20 in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a construct encoding a variant xylanase polypeptide according to the present invention and
25 which is capable of introducing the construct into the genome of a plant.

The vector system may comprise one vector, but it can comprise at least two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al.
30 (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

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One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. *et al.* (1980), *Tissue Culture*
5 *Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

10 B. Uses

In a general sense, a variant xylanase of the invention may be used to alter, for example reduce, the viscosity derived from the presence of hemicellulose or arabinoxylan in a solution or system comprising plant cell wall material. Typically said plant cell wall
15 materials will comprise one or more xylanase inhibitors.

Specifically, a variant xylanase of the invention may be used in processing plant materials for use as foodstuffs, such as animal feed, in starch production, in baking and in the processing of wood pulp to make paper.

20

Preparation of foodstuffs

A variant xylanase of the invention may be used to process plant materials such as cereals that are used in foodstuffs including animal feed. As used herein, the term "cereal" means
25 any kind of grain used for food and/or any grass producing this grain such as but not limited to any one of wheat, milled wheat, barley, maize, sorghum, rye, oats, triticale and rice or combinations thereof. In one preferred embodiment, the cereal is a wheat cereal.

The xylan in the food and/or feed supplement is modified by contacting the xylan with the
30 variant xylanase of the present invention.

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As used herein, the term "contacting" includes but is not limited to spraying, coating, impregnating or layering the food and/or feed supplement with the variant xylanase enzyme of the present invention.

5 In one embodiment, the food and/or feed supplement of the present invention may be prepared by mixing the variant xylanase enzyme directly with a food and/or feed supplement. By way of example, the variant xylanase enzyme may be contacted (for example, by spraying) onto a cereal-based food and/or feed supplement such as milled wheat, maize or soya flour.

10

It is also possible to incorporating the variant xylanase enzyme it into a second (and different) food and/or feed or drinking water which is then added to the food and/or feed supplement of the present invention. Accordingly, it is not essential that the variant xylanase enzyme provided by the present invention is incorporated into the cereal-based 15 food and/or feed supplement itself, although such incorporation forms a particularly preferred aspect of the present invention.

In one embodiment of the present invention, the food and/or feed supplement may be combined with other food and/or feed components to produce a cereal-based food and/or 20 feed. Such other food and/or feed components may include one or more other (preferably thermostable) enzyme supplements, vitamin food and/or feed supplements, mineral food and/or feed supplements and amino acid food and/or feed supplements. The resulting (combined) food and/or feed supplement comprising possibly several different types of compounds can then be mixed in an appropriate amount with the other food and/or feed 25 components such as cereal and protein supplements to form a human food and/or an animal feed.

In one preferred embodiment, the food and/or feed supplement of the present invention can be prepared by mixing different enzymes having the appropriate activities to produce 30 an enzyme mix. By way of example, a cereal-based food and/or feed supplement formed from e.g. milled wheat or maize may be contacted (e.g. by spraying) either simultaneously or sequentially with the xylanase enzyme and other enzymes having

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appropriate activities. These enzymes may include but are not limited to any one or more of an amylase, a glucoamylase, a mannanase, an a galactosidase, a phytase, a lipase, a glucanase, an-arabinofuranosidase, a pectinase, a protease, a glucose oxidase, a hexose oxidase and a xylanase. Enzymes having the desired activities may for instance be mixed with the xylanase of the present invention either before contacting these enzymes with a cereal-based food and/or feed supplement or alternatively such enzymes may be contacted simultaneously or sequentially on such a cereal based supplement. The food and/or feed supplement is then in turn mixed with a cereal-based food and/or feed to prepare the final food and/or feed. It is also possible to formulate the food and/or feed supplement as a solution of the individual enzyme activities and then mix this solution with a food and/or feed material prior to processing the food and/or feed supplement into pellets or as a mash.

Bakery products

The present invention provides the use of a variant xylanase polypeptide of the invention in a process for preparing a foodstuff. Typical bakery (baked) products in accordance with the present invention include bread - such as loaves, rolls, buns, pizza bases etc. - pretzels, tortillas, cakes, cookies, biscuits, crackers etc. The preparation of foodstuffs such as bakery products is well know in the art. Dough production, for example, is described in example 2. The use of variant xylanases of the invention to alter the viscosity of a flour slurry in described in the example 5.

Starch production

A variant xylanase of the invention may also be used in starch production from plant materials derived from cereals and tubers, such as potatoes.

Processing of wood pulp

A variant xylanase of the invention may also be used in processing wood pulp, for example in the preparation of paper.

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As discussed above, we have shown that a major determinant of xylanase functionality is the presence of endogenous inhibitors in plant material. Consequently, although one method for altering xylanase functionality is to modify a xylanase to change its sensitivity to endogenous inhibitors, another method would be to vary the amount and/or type of inhibitor present in the plant material. Thus, the present invention also provides the use of a xylanase inhibitor to alter the functionality of a xylanase and consequently the use of a xylanase inhibitor in the methods of processing plant materials described above.

The present invention will now be further described with reference to the following examples which are intended to be illustrative only and non-limiting.

EXAMPLES

Example 1 - Purification and characterisation of wheat endogenous xylanase inhibitor.

2 kg wheat flour (Danish reform, batch 99056) was extracted with water, using a flour:water ratio of 1:2, during 10 minutes of stirring. The soluble endogenous xylanase inhibitor was separated from the flour-water slurry by centrifugation. The extraction and centrifugation was performed at 4°C. The inhibitor was purified from the water extract by the following chromatographic techniques and concentration techniques: HPLC-SEC, HPLC-CIEC, rotary evaporation, HPLC-HIC, HPLC-SEC and rotary evaporation. The xylanase inhibitor could be monitored and quantified during purification, using the following quantification method.

Inhibitor quantification method

1 XIU (Xylanase Inhibitor Unit) is defined as the amount of inhibitor that decreases 1 TXU to 0.5 TXU under the conditions described below.

The xylanase used in this assay is *Bacillus subtilis* wild type xylanase.

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250 µl xylanase solution containing 12 TXU/ml, approx. 100 µl xylanase inhibitor solution and citric acid (0.1 M) - *di*-sodium-hydrogen phosphate (0.2 M) buffer, pH 5, to react a reaction volume of 1000 µl is pre-incubated for 5 minutes at 40°C. At t = 5
5 minutes, 1 Xylazyme (Megazyme, Ireland) tablet is added to the reaction mixture. At t = 15 minutes the reaction is terminated, by addition of 10 ml 2 % TRIS/NaOH, pH 12. The solution is filtered and the absorbency of the supernatant is measured at 590 nm. By choosing several different concentrations of inhibitor in the above assay, it is possible to create a plot of OD versus inhibitor concentration. Using the slope (a) and intercept (b)
10 from this plot and the concentration of the xylanase it is possible to calculate the amount of XIU in a given inhibitor solution (equation 1).

Equation 1 amount of XIU in solution = ((b/2)/-a)/ TXU

15 From the endogenous xylanase inhibitor purification, the following inhibitor yield was recovered (table 1).

Table 1. Wheat endogenous xylanase inhibitor recovery after purification.

Sample	Amount	XIU	XIU, total	Recovery, %
Flour	2000 g	590/g	1.180.000	100
Purified inhibitor	90 ml	4658/ml	419.220	35.5

20

The inhibitor sample was pure and free from wheat endogenous xylanolytic activities.

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Example 2 - Fractionation and reconstruction of wheat flour free of xylanase inhibitor and xylanases functionality in this flour as a function of added xylanase inhibitor.

5 Flour fractionation and reconstitution

The flour used was: Danish Reform flour, batch No 99056. The fractionation, inhibitor inactivation and reconstitution were as follows:

10 A simple dough was made by mixing 1600 gram flour, with optimal water addition, according to a baker's absorption at 500 BU and mixing time according to Farinograph results. This resulted in 2512 gram dough. The gluten was manually washed out from the dough, using a water dough ratio of approx. 5:1. The water used was pre-chilled to 4°C to prevent further enzyme activity in the dough. The resulting wash-water contained the
15 soluble proteins (including the xylanase inhibitor), lipids, non-starch polysaccharides and starch. The starch and other non-soluble components were separated from the wash-water by centrifugation (5000 g, 10 minutes, 10°C). To inactivate the endogenous xylanase inhibitor in the wash-water, the supernatant from the centrifugation was boiled for three minutes using a heat-evaporator.

20

All three fractions (gluten, starch and solubles) were frozen in flasks and placed in a freeze dryer. After drying, the fractions were weighed, grounded using a mortar and pestle, coffee mill and sieved through a 250 μm sieve. All fractions were weighed again and flour was reconstituted, based on the ratios obtained after fractionation.

25

Enzymes

The xylanases listed in table 2 have been used in the study. The xylanases are purified, meaning no other xylonolytic activity is present in the sample.

30

Table 2. Xylanases used in the study and activity, TXU.

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ID	Origin	TXU
B. sub	*B. subtilis.*	5100
A. nig	*A. niger*	8800

Xylanase assay (Endo-β-1,4-Xylanase activity)

Xylanase samples are diluted in citric acid (0.1 M) - *di*-sodium-hydrogen phosphate
5 (0.2 M) buffer, pH 5.0, to obtain approx. OD = 0.7 in the final assay. Three dilutions of
the sample and an internal standard with a defined activity are thermostated for 5 minutes
at 40°C. At time = 5 minutes, 1 Xylazyme tab (crosslinked, dyed xylan substrate) is
added to the enzyme solution. At time = 15 minutes (or in some cases longer, depending
on the xylanase activity present in the sample) the reaction is terminated, by adding 10 ml
10 of 2% TRIS. The reaction mixture is centrifuged and the OD of the supernatant is
measured at 590 nm. Taking into account the dilutions and the amount of xylanase, the
activity (TXU, Total-Xylanase-Units) of the sample can be calculated relative to the
standard.

15 Baking trials

Baking trials were done with (1.44 x initial inhibitor level in Danish Reform flour, batch
No 99056) and without addition of purified endogenous xylanase inhibitor to the
reconstituted flour, respectively. The baking trials were done using the xylanases listed in
20 table 2 and the compositions listed in table 3..

25

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Table 3. Composition of dough made within the baking trials.

Dough No	ID	TXU	Inh. add, XIU/50g
1	Control	0	0
2	*B. sub*	7500	0
3	*A. nig*	7500	0
4	*B. sub*	7500	850
5	*A. nig*	7500	850
6	Control	0	850

Dough analysis

5

The dough were analysed with respect to:

Stickiness
Dough stickiness was measured on a TX-XT2 system (Stable Micro Systems) using a
10 SMS Dough Stickiness Cell according to the method described by Chen And Hoseney (Lebensmittel Wiss u.- Technol., 28, 467-473. 1995).

Viscosity analysis of dough liquid
The viscosity of extracted dough liquid was measured using a Brookfield viscosimeter
15 after extraction.

Pentosan analysis of dough liquid
Solubilised pentosan was measured in the dough liquid using the method of Rouau and Surget (Carbohydrate polymers, 24, 123-132, 1994).

20

RESULTS

Flour fractionation and reconstitution

The fractionation and reconstitution of the dough resulted in 168.15 grams of freeze dried gluten, 111.13 grams of freeze dried soluble fraction and 1143.56 grams of freeze dried starch.

Inhibitor quantification in flour

Using the inhibitor quantification method, the inhibitor level in the 99056 flour and the reconstituted flour could be detected. The results from these analyses are listed in table 4.

Table 4. Results from inhibitor quantification in native flour (99056) and reconstituted flour.

Flour	Inhibitor concentration, XIU/g flour
99056	590
Reconstituted flour	42

Comparing the inhibitor level in the two portions of flour a 93% (100 - (42XIU/590XIU) x 100 %)) decrease of inhibitor level in the reconstituted flour is shown.

Baking trials

The results from the baking trial are listed in tables 5 and 6.

Table 5. Data from baking trials with reconstituted flour, xylanase and +/- xylanase inhibitor addition. Std. dev., % represents the standard deviation over two days of baking.

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ID	TXU	Inh., XIU/50g	Avg. spec. vol, ml/gram	Std. dev., %
Control	0	42	3.04	4.06
B. sub	7500	42	3.23	12.51
A. nig.	7500	42	3.44	5.24
B. sub	7500	850	3.22	4.26
A. nig.	7500	850	3.38	0.70
Control	0	850	2.94	0.05

The standard deviation shown in table 5 reflects the dough handling properties of the tested dough. The dough made without the endogenous xylanase inhibitor (42 XIU), were very difficult to handle. The standard deviation for these doughs are in the area of 3 to 12.5 %. Compared to the dough with the inhibitor added, this is quite high. If these standard deviations are compared with the actual changes in the bread volume, it can be seen that the figures are approximately the same value. This means that we can not conclude anything about the absence of the inhibitor's influence on the bread volume. If we look at the dough made with addition of the endogenous xylanase inhibitor (850 XIU) in table 5, we can see that we were able to produce bread from the reconstituted flour in a reproducible way over a two day period. The standard deviation was within the area of 0.05 to 4.2 %, which is acceptable. From table 6 it can be seen, that the xylanases all increased the volume of the baked bread.

Table 6. Volume increase in bread baked from reconstituted flour as a function of xylanase and xylanase inhibitor addition.

ID	TXU	Inh., XIU/50g	Avg. spec. vol, ml/gram	Volume increase as function of xylanase, %
Control	0	42	3.04	0.0
B. sub	7500	42	3.23	6.2
A. nig.	7500	42	3.44	13.3
B. sub	7500	850	3.22	9.7
A. nig.	7500	850	3.38	15.0
Control	0	850	2.94	0.0

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What can be deduced from table 5 and table 6, is that the absence of the xylanase inhibitor in the flour made the handling of the dough very difficult. Therefore, what may seem as a positive response in volume by addition of inhibitor in table 6, probably can be explained by the high standard deviation in the dough lacking the inhibitor, due to difficult handling properties. Furthermore, it can be concluded that all the xylanases tested increased the bread volume significantly compared to the blank control.

Stickiness

The same dough, that was used for the baking trials, was used for stickiness measurements. The results are listed in table 7.

Table 7. Data representing stickiness as a function of time, xylanase and xylanase inhibitor addition to reconstituted flour.

ID	TXU	Inh., XIU/50g	Avg. stickiness after 10 min, g x s	Avg. stickiness after 60 min, g x s
Control	0	42	4.71	4.79
B. sub.	7500	42	12.20	13.39
A. nig.	7500	42	9.22	12.58
B. sub.	7500	850	2.51	3.66
A. nig.	7500	850	5.24	6.45
Control	0	850	4.10	4.15

The results in table 7 clearly indicate the influence of the inhibitor that was observed in the experiment. The dough with a low level of xylanase inhibitor in combination with xylanase, was very difficult to handle and mould. However, when the inhibitor was added, the dough became dry and very easy to handle. As can be seen from table 7, addition of the 990202 xylanase in combination with the inhibitor decreased the stickiness. The dough became drier.

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Table 7 also shows that there is only a small effect of time on the stickiness. It seems that the xylanases act very rapidly. Within the first 10 minutes most of the arabinoxylan is modified when the first xylanase (*B. sub*) is added. The second xylanase tested (*A. nig*), seems to act less rapidly. A function of time can easily be observed using this xylanase. This is also the xylanase that shows the least effect as a function of inhibitor level when analysed on stickiness.

Dough Viscosity

The dough viscosity and the pentosan analysis results were obtained from the same extraction of dough prepared from reconstituted flour added xylanase and xylanase inhibitor. This dough was analysed after two proofing times, 30 and 120 minutes.

The results of the viscosity analysis are presented in table 8.

Table 8. Data representing dough liquid viscosity as a function of time, xylanase and xylanase inhibitor addition to reconstituted flour.

ID	TXU	Inh., XIU/50g	Avg. dough viscosity, cP, 30 min proofing	Avg. dough viscosity, cP, 120 min proofing
Control	0	42	5.21	5.56
B. sub.	7500	42	5.07	4.55
A. nig.	7500	42	5.78	4.14
B. sub.	7500	850	9.03	11.09
A. nig.	7500	850	8.44	8.55
Control	0	850	5.96	6.95

As can be seen from table 8 the inhibitor has a significant effect on the functionality of the xylanases. Without addition of the inhibitor, the arabinoxylan is being de-polymerised to Low Molecular Weight (LMW) arabinoxylan with a low viscosity. Addition of inhibitor prevents this very extensive de-polymerisation of the arabinoxylan.

Pentosan analysis of dough liquid

The results from the pentosan (arabinoxylan) analysis of the dough liquid are presented in table 9.

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Table 9. Data representing pentosan solubilisation as a function of time, xylanase and xylanase inhibitor addition to reconstituted flour.

ID	TXU	Inh., XIU/50g	Avg. Pentosan, %, 30 min proofing	Avg. Pentosan, %, 120 min proofing
Control	0	42	0.387	0.458
B. sub.	7500	42	0.766	0.819
A. nig.	7500	42	0.719	0.798
B. sub.	7500	850	0.410	0.544
A. nig.	7500	850	0.560	0.673
Control	0	850	0.400	0.528

5 As can be seen from the results in table 9, the addition of endogenous xylanase inhibitor decreased the solubilisation of the arabinoxylan. When evaluated after 30 minutes proofing time, the amount of arabinoxylan solubilised in absence of the inhibitor is almost twice the amount as in presence of the inhibitor. Calculated on the basis of the relating control samples, the solubilisation is much higher in absence of the inhibitor, as illustrated
10 in the following example:

$$(0.766-0.387)/(0.410-0.400) = 37.9 \text{ times higher solubilisation}$$

The above example was calculated on basis of solubilisation of arabinoxylan using the
15 *Bacillus* xylanase, 30 minutes proofing and +/- inhibitor.

Example 3 - Site-directed mutagenesis on xylanases.

Specific mutants of *Bacillus subtilis* xylanase may be obtained by site directed
20 mutagenesis of the wild type enzyme, by the use of any of a number of commercially available mutagenesis kits. An example of how to obtain the D11F mutant using the Quick Exchange kit, available from Stratagene Cloning Systems, 11011 North Torrey Pines Road, La Jolla, CA 92037, USA is given below :

25 The DNA sequence encoding *Bacillus subtilis* xylanase A has been published by Paice et al., 1986.

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The sequence of the coding region is as follows, with the sequence encoding the mature part of the protein shown in capitals:

```
catatgtttaagtttaaaaagaatttcttagttggattatcggcagctttaatgagtatt
agcttgttttcggcaaccgcctctgcaGCTAGCACAGACTACTGGCAAAATTGGACTGAT
GGGGGCGGTATAGTAAACGCTGTCAATGGGTCTGGCGGGAATTACAGTGTTAATTGGTCT
AATACCGGAAATTTTGTTGTTGGTAAAGGTTGGACTACAGGTTCGCCATTTAGGACGATA
AACTATAATGCCGGAGTTTGGGCGCCGAATGGCAATGGATATTTAACTTTATATGGTTGG
ACGAGATCACCTCTCATAGAATATTATGTAGTGGATTCATGGGGTACTTATAGACCTACT
GGAACGTATAAAGGTACTGTAAAAAGTGATGGGGGTACATATGACATATATACAACTACA
CGTTATAACGCACCTTCCATTGATGGCGATCGCACTACTTTTACGCAGTACTGGAGTGTT
CGCCAGTCGAAGAGACCAACCGGAAGCAACGCTACAATCACTTTCAGCAATCATGTGAAC
GCATGGAAGAGCCATGGAATGAATCTGGGCAGTAATTGGGCTTACCAAGTCATGGCGACA
GAAGGATATCAAAGTAGTGGAAGTTCTAACGTAACAGTGTGGTAA
```

The part of the gene encoding the mature part of the wild type enzyme may be expressed intracellularly in *E.coli* by methods well known to people skilled in the art of molecular biology. For example :

1. Generating a copy of the capitalised part of the above described gene by use of the Polymerase Chain Reaction (PCR) with an added Nde1 restriction enzyme site (CATATG) before the GCTAGCACA and an added HindIII restriction site (AAGCTT) after the GTGTGGTAA.
2. Inserting the resultant modified copy of the gene by use of the above mentioned enzymes into the expression vector pET24a(+), which can be obtained from Novagen, Inc. 601 Science Drive, Madison, WI 53711, USA.
3. Transforming into a suitable *E.coli* strain and expression by fermentation as described by the vendor of pET24a(+).

Our D11F mutant enzyme may be obtained by using the "Quick Exchange" mutagenesis kit according to the manufacturer, and using the above described *Bacillus subtilis* wild type xylanase-pET24a(+) construct and the following PCR mutagenesis primers :

Sense primer:

CTACTGGCAAAATTGGACTTTTGGAGGAGGTATAGTAAACGCTG

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Antisense primer :
CAGCGTTTACTATACCTCCTCCAAAAGTCCAATTTTGCCAGTAG

5 The mutant enzyme is expressed and purified using the same protocols as for the wild type enzyme.

Example 4 - Inhibition studies of xylanase mutants.

10 Xylanase mutants expressed in *E. coli* (see Example 3) were fermented and purified (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

These pure xylanase mutant preparations were diluted to 12 TXU/ml using 0.1 M citric 15 acid - 0.2 M *di*-sodium-hydrogen phosphate, pH 5.0 and used in the following assay.

A stable inhibitor preparation was made according to the protocol described in Example 1. This stable inhibitor preparation is used as stock for all xylanase-xylanase inhibitor studies. Using the inhibitor quantification method described in example 1, the inhibitor 20 preparation was analysed to contain 126 XIU/ml.

Assay

To 250 µl diluted xylanase mutant preparations, are added 0, 10, 25, 50 or 100 µl inhibitor preparation, respectively. To these inhibitor-xylanase mixtures were added 0.1 M citric 25 acid - 0.2 M *di*-sodium-hydrogen phosphate, pH 5.0 making the end-volume 1000 µl. These reaction mixtures were pre-incubated for 5 minutes at 40°C. Hereafter 1 xylazyme tablet (Megazyme, Ireland) were added to all inhibitor-xylanase mixtures. After 10 minutes of incubation at 40°C, the reactions were terminated, by adding 10 ml 2% Tris/NaOH, pH 12.0. The mixtures were centrifuged and the liberated blue colour from 30 the substrate was measured at 590 nm.

The results are presented in table 10.

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Table 10. Relative inhibition of xylanase mutants and parent xylanase (here wildtype enzyme) as a function of xylanase inhibitor.

Mutant ID	0	1,26	3,15	6,3	12,6
			Relative inhibition, %		
Wildtype	100	77	48	29	23
D11Y	100	120	114	126	124
D11N	100	93	72	53	32
D11F	100	114	119	116	115
D11K	100	109	112	113	116
D11S	100	98	81	60	38
D11W	100	101	88	70	50
G34D	100	94	83	70	53
G34F	100	76	53	34	29
G34T	100	99	99	93	86
Y113A	100	96	80	62	43
Y113D	100	96	81	63	45
Y113K	100	103	85	63	47
N114A	100	80	49	28	22
N114D	100	84	57	39	29
N114F	100	84	54	39	34
N114K	100	87	56	33	24
D121N	100	80	36	16	14
D121K	100	104	95	85	75
D121F	100	101	89	72	60
D121A	100	81	50	27	21
R122D	100	85	59	41	28
R122F	100	93	74	58	58
R122A	100	78	46	33	26
Q175E	100	87	59	40	31
Q175S	100	88	59	30	19
Q175L	100	78	42	25	23
G12F	100	110	106	100	92
G13F	100	104	95	87	84
I15K	100	84	47	28	23
N32K	100	82	42	19	14
G120K	100	85	52	29	22
G120D	100	84	47	24	18
G120F	100	71	35	18	15
G120Y	100	81	40	18	16
G120N	100	84	49	29	23
D119K	100	94	67	40	26
D119Y	100	87	50	28	22
D119N	100	91	74	44	22
T123K	100	80	46	30	25
T123Y	100	80	47	28	27
T123D	100	83	36	20	17
T124K	100	110	92	73	57
T124Y	100	101	76	49	33
T124D	100	87	52	32	25

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N17K	100	88	48	31	26
N17Y	100	79	42	23	19
N17D	100	90	81	50	22
N29K	100	83	50	30	23
N29Y	100	85	49	30	24
N29D	100	74	44	26	20
S31K	100	77	42	23	23
S31Y	100	83	50	27	22
S31D	100	79	52	30	24
D11F/R122D	100	109	111	110	109
D11F/G34D	100	104	106	103	104

From the results in table 10, it can be seen the xylanase mutants D11Y, D11F, D11K, D11F/R122D and D11F/G34D are uninhibited by the wheat endogenous xylanase inhibitor. These xylanase mutants would be expected to act more aggressively/specifically on the soluble arabinoxylan, compared to the other xylanase mutants or other xylanases. They would therefore be superior in applications where a decrease in viscosity (as a function of HMW arabinoxylan) is wanted.

Example 5 - Functionality studies of xylanase mutants.

Xylanase mutants expressed in *E. coli* (see Example 3) were fermented and purified (meaning no other xylanolytic activity were present in the purified preparation).

These pure xylanase mutant preparations were diluted to 400 TXU/ml using water and used in the following assay.

Assay

200 ml 30 % (w/w) flour slurry was made using water (thermostated to 25°C), by stirring for 5 minutes. 60.0 ml of this flour slurry is poured into a Ford-cup, and the time for drainage of 50.0 ml is measured. This measurement is the blank measurement. The 60.0 ml flour slurry is poured back and 1000 µl diluted xylanase mutant preparation is added to the flour slurry under stirring. After 2, 5, 10 and 20 minutes, 60.0 ml is poured into the Ford-cup, and the drainage time for 50.0 ml is recorded. Each measurement were done in triplicate.

The results are presented in table 11.

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Table 11. Relative viscosity of flour slurry as a function of xylanase mutant and parent xylanase (here wild type xylanase)

		Incubation time, minutes			
Mutant ID	0	2	5	10	20
		Relative viscosity change, %			
Wildtype	100	112	120	131	141
D11Y	100	97	93	83	75
D11N	100	112	125	130	136
D11F	100	93	87	78	69
D11K	100	105	95	88	78
D11S	100	102	110	113	117
D11W	100	106	115	121	122
G34D	100	110	120	128	124
G34F	100	111	126	128	146
G34T	100	100	108	111	106
Y113A	100	118	129	130	124
Y113D	100	116	127	124	114
Y113K	100	118	123	121	115
N114A	100	117	128	127	131
N114D	100	125	144	162	170
N114F	100	113	119	131	150
N114K	100	119	129	141	147
D121N	100	104	103	106	104
D121K	100	122	132	141	162
D121F	100	107	117	128	147
D121A	100	101	102	103	107
R122D	100	120	119	124	115
R122F	100	127	144	150	160
R122A	100	123	138	144	153
Q175E	100	116	134	142	149
Q175S	100	110	113	121	129
Q175L	100	111	111	119	126
G12F	100	127	132	122	101
G13F	100	106	119	124	113
I15K	100	109	108	113	118
N32K	100	97	98	101	101
G120K	100	103	111	115	121
G120D	100	112	122	120	126
G120F	100	103	111	117	130
G120Y	100	106	106	108	126
G120N	100	119	123	130	141
D119K	100	118	119	127	125
D119Y	100	102	102	111	110
D119N	100	126	137	145	146
T123K	100	106	109	121	120
T123Y	100	101	106	108	116
T123D	100	113	123	125	126
T124K	100	117	131	128	127
T124Y	100	112	123	132	135

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T124D	100	103	110	111	118
N17K	100	114	119	119	132
N17Y	100	102	102	108	108
N17D	100	120	131	135	143
N29K	100	98	100	100	104
N29Y	100	115	117	132	143
N29D	100	104	104	113	111
S31K	100	119	115	124	134
S31Y	100	110	118	122	137
S31D	100	99	103	109	110
D11F/R122D	100	91	89	82	77
D11F/G34D	100	96	93	84	80

Example 6. Site-directed mutation in the active site of *Bacillus subtilis* xylanase A, does not influence the xylanase : xylanase inhibitor interaction.

5

A residue in the active site of the *Bacillus subtilis* wildtype xylanase A enzyme was altered by a site-directed mutation (see ex. 3) In the mutated residue (Y166F) a potential hydrogen bond is lost. The mutant xylanase, was expressed in *E. coli*, fermented and purified. Hereafter, the mutant was investigated for its interaction with the xylanase
10 inhibitor (see example 4).

As can be seen below (table 12), the exchange of an amino acid in the active site, did surprisingly not have any effect on interactions with the xylanase inhibitor as compared to the *Bacillus subtilis* wildtype xylanase enzyme.

15

Table 12. Relative inhibition of *Bacillus subtilis* wildtype xylanase and the xylanase mutant Y166F.

			XIU/ml		
Xylanase ID	0	1,26	3,15	6,3	12,6
			Relative inhibition, %		
Wildtype	100	75	40	24	20
Y166F	100	74	39	22	20

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Hence, in summary the experiment described above shows a site-directed mutation in the active site of the Bacillus subtilis xylanase A, which mutation does not influence the xylanase's interactions with the xylanase inhibitor.

5 **Example 7. Site-directed mutation in family 11 xylanases other than the *Bacillus subtilis* xylanase A, influencing the xylanase - xylanase inhibitor interactions.**

D19 residue of the *Thermomyces lanuginosus* xylanase A enzyme was mutated to F19 by
10 site-directed mutagenesis. D19 corresponds to D11 residue in the *Bacillus subtilis* xylanase (SEQ ID NO. 1). *Thermomyces lanuginosus* xylanase A gene is described as SEQ ID NO. 9.

The primers for PCR construction of the D19F mutant may be the following :

15

Sense primer :
GGTTATTACTATTCCTGGTGGAGTTTTGGAGGAGCGCAGGCCACG
Antisense primer :
CGTGGCCTGCGCTCCTCCAAAACTCCACCAGGAATAGTAATAACC

20

The obtained mutant xylanase (D19F), was expressed in *E. coli*, fermented and purified. Hereafter, the mutant and the *Thermomyces lanuginosus* wildtype xylanase A was investigated for to its interaction with the xylanase inhibitor (see example 4). As can be seen from the results in table 13, the D19F mutant of the *Thermomyces lanuginosus*
25 xylanase A is significantly less inhibited by the xylanase inhibitor as compared to the *Thermomyces lanuginosus* wildtype xylanase A.

Table 13. Relative inhibition of *Thermomyces lanoginosus* wildtype xylanase A (TLX) and the *Thermomyces lanoginosus* mutant xylanase, D19F (D19F).

30

			XIU/ml		
Xylanase ID	0	1,26	3,15	6,3	12,6
			Relative inhibition, %		
TLX	100	45	24	17	14
D19F	100	73	38	24	20

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Hence, in summary the experiment described above shows a site-directed mutation in the *Thermomyces lanuginosus* xylanase A. The results show that a mutation introducing a substitution of an amino acid on the surface of the xylanase molecule (analogue to the
5 D11F in *B. subtilis*) changes the xylanase:xylanase inhibitor interactions. Thus, our invention (i.e. that surface residues control the level of inhibition of xylanase) holds true for xylanases that are homologous to the *B. subtilis* xylanase.

SUMMARY

10

In summary, the present invention provides a means for altering the sensitivity of a xylanase enzyme to a xylanase inhibitor.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been
15 described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

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15

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CLAIMS

1. A variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor as compared with the parent xylanase enzyme.

2. A variant polypeptide according to claim 1 which is derived from a family 11 xylanase.

3. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to claim 1 or claim 2 wherein said amino acid modification is of one or more surface amino acid residues.

4. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to any one of the preceding claims wherein said amino acid modification is of one or more solvent accessible residues.

5. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to any one of the preceding claims wherein there are at least two of said amino acid modifications.

6. A variant xylanase polypeptide, or fragment thereof having xylanase activity according to any one of the preceding claims wherein said amino acid modification is at any one or more of amino acid residues:

5 Ala1-Trp6, Asn8, Thr10-Gly23, Asn25, Ser27, Asn29, Ser31-Asn32, Gly34, Thr43-Thr44, Ser46-Thr50, Asn52, Asn54, Gly56-Asn61, Asn63, Arg73-Leu76, Thr87-Arg89, Thr91-Lys95, Thr97, Lys99, Asp101-Gly102, Thr104, Thr109-Thr111, Tyr113-Asn114, Asp119-Thr124, Thr126, Gln133-Asn141, Thr143, Thr145, Thr147-Asn148, Asn151, Lys154-Gly157, Asn159-Leu160, Ser162-
10 Trp164, Gln175, Ser177, Ser179, Asn181, Thr183, Trp185

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of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or its/their equivalent positions in other homologous xylanase polypeptides.

7. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to any one of the preceding claims wherein said amino acid modification is at any one or more of amino acid residues numbers: 11, 12, 13, 15, 17, 29, 31, 32, 34, 113,
5 114, 119, 120, 121, 122, 123, 124 and 175 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

8. A variant xylanase polypeptide, or fragment thereof having xylanase activity,
10 according to claim 7 wherein said variant xylanase polypeptide, or fragment thereof having xylanase activity, in addition comprises one or more amino acid modifications at any one of the other amino acid residues.

9. A variant xylanase polypeptide, or fragment thereof having xylanase activity,
15 according to claim 8 wherein said other amino acid residues are any one or more of amino acid residues numbers: 3, 4, 5, 6, 7, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 38, 39, 40, 41, 42, 43, 44, 45, 55, 56, 57, 58, 59, 60, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 108, 109, 110, 126, 127, 128, 129, 130, 131, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171,
20 172, 179, 180, 181, 182, 183 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

10. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to claim 8 wherein said other surface amino acid residues are any one or more
25 of amino acid residues numbers: 1, 2, 46, 47, 48, 49, 50, 51, 52, 53, 54, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 184, 185 of the *B. subtilis* amino acid sequence shown as SEQ I.D. No. 1 or their equivalent positions in other homologous xylanase polypeptides.

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11. A variant polypeptide according to any one of the preceding claims wherein the inhibitor is an inhibitor found naturally in plant tissues.

12. A variant polypeptide according to any one of the preceding claims wherein the sensitivity to the inhibitor is reduced.

13. A method of altering the sensitivity of a xylanase polypeptide to an inhibitor which method comprises modifying one or more amino acid residues of said enzyme such that the polypeptide or a fragment thereof has an altered sensitivity to a xylanase inhibitor as compared with the parent xylanase enzyme.

14. A method according to claim 13 wherein said variant polypeptide is that defined in any one of claims 1 to 12.

15. A method according to claim 13 or claim 14 wherein the sensitivity is reduced.

16. A composition comprising a variant polypeptide according to any one of claims 1 to 12.

17. A method of degrading or modifying a plant cell wall which method comprises contacting said plant cell wall with a polypeptide according to any one of claims 1 to 12 or a composition according to claim 16.

18. A method of processing a plant material which method comprises contacting said plant material with a polypeptide according to any one of claims 1 to 12 or composition according to claim 16.

19. A nucleotide sequence encoding a variant polypeptide according to any one of claims 1 to 12.

20. A construct comprising the nucleotide sequence according to claim 19.

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21. Use of a variant polypeptide according to any one of claims 1 to 12 in a method of modifying plant materials.

22. Use of a variant polypeptide according to any one of claims 1 to 12 in any one or more of: baking, processing cereals, starch production, in processing wood, enhancing the bleaching of wood pulp.

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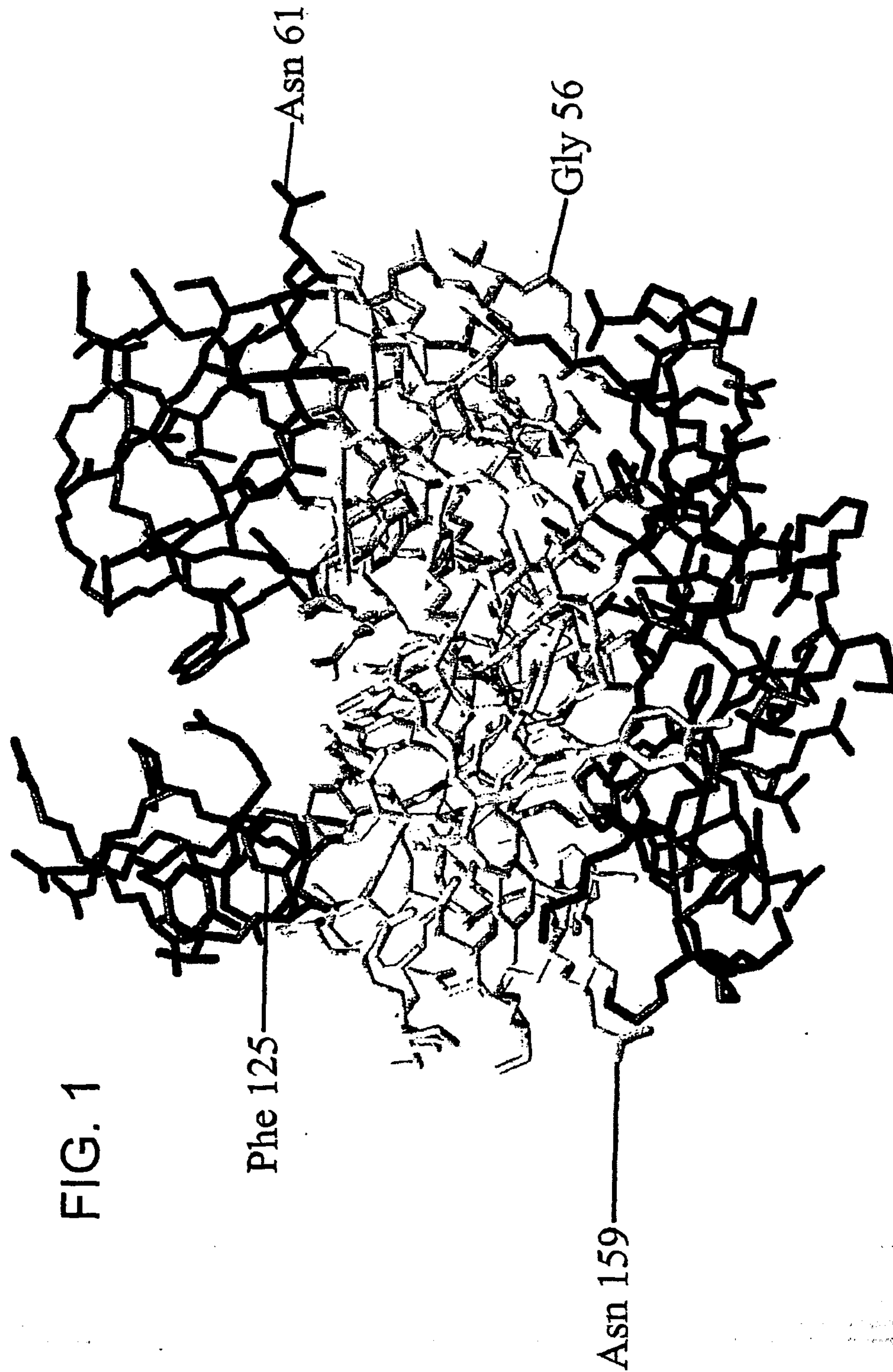

FIG. 1

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FIG. 2

```
                               1             15 16          30 31          45 46          60 61          75 76          90
B. subtilis xylA               --------------- ----MFKFKKNFLVG LSAALMSISLFSATA S-------------- --------------- ---------AASTDY   33
B. circulans xylA              --------------- ----MFKFKKNFLVG LSAALMSISLFSATA S-------------- --------------- ---------AASTDY   33
B. stearothermophilus xylA     --------------- -----MKLKKKMLTL LLTASMSFGLFGATS --------------- --------------- ---------SAATDY   31
A. caviae xylA                 --------------- ----MFKFGKKLMTV VLAASMSFGVFAATS --------------- --------------- ---------SAATDY   32
C. carbonum xyl1               --------------- ----MVSFTSIITAA VAATGALAAPATDVS --------------- ------------LVA RQNTPNGEGTHNGCF   44
H. turcicum xyl1               --------------- ----MVSFTSIITAA VAATGALAAPATDIA ARAP----S------ -----------DLVA RQSTPNGEGTHNGCF   50
A. pisi xyl                    --------------- ----MVSFTSIFTAA VAATGALAVPVTDLA TRSL----G------ -----------ALTA RAGTPSSQGTHNGCF   50
S. commune xylA                --------------- --------------- --------------- --------------- --------------A ASGTPSSTGTDGGYY   16
T. lanuginosus xylA            --------------- ----MVGFTPVALAA LAATGALAFPAGNAT --------------- -----------ELEK RQTTPNSEGWHDGYY   45
C. carbonum xyl2               --------------- ----MVSFKSLLLAA VATTSVLAAPFDFLR ERDDVNATA------ -----------LLEK RQSTPSAEGYHNGYF   54
C. sativus xyl2                --------------- ----MVSFKSLLLAA VATTSVLAAPFDFLR ERDDGNATA------ -----------LLEK RQSTPSSEGYHNGYF   54
H. insolens xyl1               --------------- ----MVSLKSVLAAA TAVSSAIAAPFDFVP RDN----ST------ -----------ALQA RQVTPNAEGWHNGYF   50
M. grisea xyl22               --------------- ----MVSFTSIVTAV VALAGSALAIPAPDG NMTG-FPFE------ -----------QLMR RQSTPSSTGRHNGYY   53
C. gracile cgxA                --------------- ----MVSFKALLLGA AG----ALAFPFNVT QMN------------ -----------ELVA RAGTPSGTGTNNGYF   44
T. reesei xyl2(2)              --------------- ----MVSFTSLLAGV AAISGVLAAPAAEVE PV------------- -----------AVEK RQTIQPGTGYNNGYF   47
T. reesei, ALKO2721 Xyl2       --------------- ----MVSFTSLLAGV AAISGVLAAPAAEVE SV------------- -----------AVEK RQTIQPGTGYNNGYF   47
T. reesei xyl1                 --------------- ----MVSFTSLLAAS PPSR-ASCRPAAEVE SV------------- -----------AVEK RQTIQPGTGYNNGYF   46
T. harzianum xylD              --------------- --------------- --------------- --------------- --------------- -QTIGPGTGYSNGYY   14
T. viride xyl                  --------------- ----MVSFTTLLAGF VAVTGVLSAPTETVE VV------------- -----------DVEK RQTIGPGTGFNNGYY   47
C. gracile cgxB                --------------- ----MVNFSSLFLAA SAAVVAVAAPGELPG --------------- -----------MHKR QTLTSSQTGTNNGYY   45
A. niger xyl2                  --------------M LTKNLLLCFAAAKAA LAVPHDSVAQRS-DA L-------------- -----------HMLS ERSTPSSTGENNGFY   50
Penicillium sp 40 xylA         -------------MK SFIAYLLASVAVTGV MAVPG-EYHKR---- --------------- -----------HDKR QTITSSQTGTNNGYY   46
Streptomyces sp xyl            MQQDGKRQDQNQQNP APFSGLSRRGFLGGA GTVALATASGLLLPS T-------------- -----------AHAA TTITTNQTGYDG-MY   64
Streptomyces sp xyl1           ----------MTKDN TPIRPVSRRGFIGRA GALALATSG-LMLPG T-------------- -----------ARAD TVITTNQTGTNNGYY   54
S. thermocyaneoviolaceus xylB  -------MNTLVHPQ GRAGG--LRLLVRAA WALALAALAAMMFGG T-------------- -----------ARAD -TITSNQTGTHNGYF   55
S. viridosporus T7A svxA       -------MNAFAHPR GRRHGRSAPMSPRST WAVLLAALAVMLLPG T-------------- -----------ATAA PVITTNQTGTNNGWW   58
T. fusca xyl                   -------MNHAPASL KSRRRFRPRLLIGKA FAAALVAVVTMIPST A-------------- -----------AHAA --VTSNETGYHDGYF   56
C. pachnodae xyl11A            ----MTRTISRAAHR PPAGGRIARALAAAG ATVAMVIAG----VA A-------------- -----------AQPA AAVDSNSTGSSGGYF   57
A. oryzae XylG1                --------------- ----MVSFSSLLLAV SAVSGALAAPGDSTL V-------------- -----------ELAK RAITSSETGTNNGYY   46
C. purpurea xyl1               --------------- ----MFLTSVVSLVV GAISCVSAAPAAAS- --------------- -------------EL MQMTPRNSCYGGGLY   42
C. mixtus xyl                  --------------- -----MKFPLIGKST LAALFCSALLGVNNT --------------- -------------QA QTLTNNATGTHNGFY   42
P. fluorescens cellulosa xyl   --------------- -----MKLPTLGKCV VRTLMGAVALGAISV --------------- -------------NA QTLSSNSTGTNNGFY   42
P. cochleariae xyl             --------------- --------------M QFLIPVVILCVSLVD --------------- -------------SQ KVLYNNEIGFNNGFY   33
A. kawachi xylC                --------------- --------------- --------------- --------------- --------------- -----------AGIN    4
A. niger xyl1                  --------------- ----MKVTAAFAGLL VTAFAAPVPEPVLVS RS------------- --------------- -----------AGIN   32
A. tubigensis xyl1             --------------- ----MKVTAAFAGLL VTAFAAPAPEPDLVS RS------------- --------------- -----------AGIN   32
P. purpurogenum xylB           --------------- ----MKVTAAFAGLL ARHSPPLSTELVTRS --------------- --------------- -------------IN   28
Cryptococcus sp S-2 xyl-CS2    --------------- ----MVASAAPVTEA EDGQAATPIAIEKRT G-------------- --------------- --------------N   28
T. reesei xyl2                 ------------MVA FSSLICALTSIASTL AMPTGLEPESSVNVT ERG------------ -----------MYDF VLGAHNDHRRRASIN   55
B. pumilus xylA(1)             --------------- MNLKRLRLLFVMCIG FVLTLTAVP------ --------------- ------------AHA ETIYDNRIGTHSGYD   42
B. pumilus xylA(2)             --------------- MNLRKLRLLFVMCIG LTLILTAVP------ --------------- ------------AHA RTITNNEMGNHSGYD   42
C. acetobutylicum XylB         --------------- -MLRRKVIFTVLATL VMTSLTIVDNTAFAA TNLNTTESTFSKEVL STQKTYSAFNTQAAP KTITSNEIGVNGGYD   74
C. thermocellum XylB           --------------- -MKQKLLVTFLILIT FTVSLTLFPVN---- --------------- -----------VRAD VVITSNQTGTGGGYN   44
Bacillus sp 41M-1 xylJ         --------------- --MKQVKIMFLMTMF LGIGLLFFSEN---- --------------- -------------AE AAITSNEIGTHDGYD   41
P. multivesiculatum xylA       --------------- --------EKVICLL IALFGLIEA------ --------------- --------------- QTFYNNAQGQIDGLD   31
P. multivesiculatum xyl        --------------- --------------- --------------- --------------- --------------- ---------------    0
R. albus xylA                  ----------MRNNF KMRVMAGVAAVICLA GVLGSCGNSSDKDSS SKKSADSAK-ADSN- ---------KDSKNG QVFTKNARGTSDGYD   69
Caldicellulosiruptor sp xylD   --------------- -------------MC VVLANPFYAQA---- --------------- --------------A MTFTSNATGTYDGYY   29
D. thermophilum xynB           --------------- MFLKKLSKLLLVVLL VAVYTQVNAQT---- --------------- --------------S ITLTSNASGTFDGYY   42
R. flavefaciens xylA           --------------- --MKLSKIKKVLSGT VSALMIASAAPVVAS --------------- --------------- -AADQQTRGNVGGYD   42
P. stipitis xylA               ---------MTVYKR KSRVLIAVVTLLHVL SHAPTKMLTTDVLLT RCMHLCHFRTSDSVY TN---------ETSE ERSMSDRLNITRVMS   72
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FIG. 2CONT'D

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                                  91          105 106         120 121         135 136         150 151         165 166         180
B. subtilis xylA                  WQNWTDGGGIVNAVN GSGGNYSVNWS--NT GNFVVGKGWTTG--- --SPFRTINYNAGVW APNGNG--YLTLYGW TRS-PLIEYYVVDSW  113
B. circulans xylA                 WQNWTDGGGIVNAVN GSGGNYSVNWS--NT GNFVVGKGWTTG--- --SPFRTINYNAGVW APNGNG--YLTLYGW TRS-PLIEYYVVDSW  113
B. stearothermophilus xylA        WQYWTDGGGMVNAVN GPGGNYSVTWQ--NT GNFVVGKGWTVG--- --SPNRVINYNAGIW EPSGNG--YLTLYGW TRN-ALIEYYVVDSW  111
A. caviae xylA                    WQNWTDGGGTVNAVN GSGGNYSVSWQ--NT GNFVVGKGWTYG--- --TPNRVVNYNAGVF APSGNG--YLTFYGW TRN-ALIEYYVVDSW  112
C. carbonum xyl1                  WSWWSDGGARATYTN GAGGSYSVSWG--SG GNLVGGKGWNP---- ---GTARTITYSGTY NYNGNS--YLAVYGW TRN-PLVEYYVVENF  122
H. turcicum xyl1                  YSWWSDGGARATYTN GAGGSYSVSWG--TG GNLVGGKGWNP---- ---GTARTITYSGQY NPNGNS--YLAIYGW TRN-PLVEYYVVENF  128
A. pisi xyl                       YSWWTDGGAQATYTN GAGGSYSVNWK--TG GNLVGGKGWNP---- ---GAARTITYSGTY SPSGNS--YLAVYGW TRN-PLIEYYVVENF  128
S. commune xylA                   YSWWTDGAGDATYQN NGGGSYTLTWSG-NN GNLVGGKGWNP---- --GAASRSISYSGTY QPNGNS--YLSVYGW TRS-SLIEYYIVESY   96
T. lanuginosus xylA               YSWWSDGGAQATYTN LEGGTYEISWG--DG GNLVGGKGWNP---- --GLNARAIHFEGVY QPNGNS--YLAVYGW TRN-PLVEYYIVENF  124
C. carbonum xyl2                  YSWWTDGGGSAQYTM GEGSRYSVTWR--NT GNFVGGKGWNP---- ---GSGRVINYGGAF NPQGNG--YLAVYGW TRN-PLVEYYVIESY  132
C. sativus xyl2                   YSWWTDGGGSAQYTM GEGSRYSVTWR--NT GNFVGGKGWNP---- ---GTGRVINYGGAF NPQGNG--YLAVYGW TRN-PLVEYYVIESY  132
H. insolens xyl1                  YSWWSDGGGQVQYTN LEGSRYQVRWR--NT GNFVGGKGWNP---- ---GTGRTINYGGYF NPQGNG--YLAVYGW TRN-PLVEYYVIESY  128
M. grisea xyl22                   YSWWTDGASPVQYQN GNGGSYSVQWQ--SG GNFVGGKGWMP---- ---GGSKSITYSGTF NPVNNGNAYLCIYGW TQN-PLVEYYILENY  133
C. gracile cgxA                   YSFWTDGGGTVNYQN GAGGSYSVQWQ--NC GNFVGGKGWNP---- ---GAARTINFSGTF SPQGNG--YLAIYGW TQN-PLVEYYIVESF  122
T. reesei xyl2(2)                 HSYWNDGHGGVTYTN GPGGQFSVNWS--NS GNFVGGKGWQP---- --GTKNKVINFSGSY NPNGNS--YLSVYGW SRN-PLIEYYIVGNF  126
T. reesei, ALKO2721 Xyl2          YSYWNDGHGGVTYTN GPGGQFSVNWS--NS GNFVGGKGWQP---- --GTKNKVINFSGSY NPNGNS--YLSVYGW SRN-PLIEYYIVENF  126
T. reesei xyl1                    YSYWNDGHGGVTYTN GPGGQFSVNWS--NS GNFVGGKGWQP---- --GTKNKVINFSGSY NPNGNS--YLSVYGW SRN-PLIEYYIVENF  125
T. harzianum xylD                 YSYWNDGHAGVTYTN GGGGSFTVNWS--NS GNFVAGKGWQP---- --GTKNKVINFSGSY NPNGNS--YLSIYGW SRN-PLIEYYIVENF   93
T. viride xyl                     YSYWNDGHSGVTYTN GAGGSFSVNWA--NS GNFVGGKGWNP---- --GSSSRVINFSGSY NPNGNS--YLSVYGW SKN-PLIEYYIVENF  126
C. gracile cgxB                   YSFWTDGQGNVQYTN EAGGQYSVTWS--GN GNWVGGKGWNP---- --GS-ARTINYTANY NPNGNS--YLAVYGW TRN-PLIEYYVVENF  123
A. niger xyl2                     YSFWTDGGGDVTYTN GDAGAYTVEWS--NV GNFVGGKGWNP---- ---GSAQDITYSGTF TPSGNG--YLSVYGW TTD-PLIEYYIVESY  128
Penicillium sp 40 xylA            YSFWTNGGGTVQYTN GAAGEYSVTWE--NC GDFTSGKGWST---- ---GSARDITFEGTF NPSGNA--YLAVYGW TTS-PLVEYYILEDY  124
Streptomyces sp xyl               YSFWTDGGGSVSMTL NGGGSYSTQWT--NC GNFVAGKGWGN---- ---GGRRTVRYSGYF NPSGNG--YGCLYGW TSN-PLVEYYIVDNW  142
Streptomyces sp xyl1              YSFWTDGGGSVSMNL ASGGSYGTSWT--NC GNFVAGKGWAN---- ---GARRTVNYSGSF NPSGNA--YLTLYGW TAN-PLVEYYIVDNW  132
S. thermocyaneoviolaceus xylB     YSFWTDAPGTVTMNT GAGGNYSTQWS--NT GNFVAGKGWAT---- ---GGRRTVTYSGTF NPSGNA--YLALYGW SQN-PLVEYYIVDNW  133
S. viridosporus T7A svxA          YSFWTDAQGTVSMDL GSGGTYSTQWR--NT GNFVAGKGWST---- ---GGRKTVNYSGTF NPSGNA--YLTLYGW TTG-PLIEYYIVDNW  136
T. fusca xyl                      YSFWTDAPGTVSMEL GPGGNYSTSWR--NT GNFVAGKGWAT---- ---GGRRTVTYSASF NPSGNA--YLTLYGW TRN-PLVEYYIVESW  134
C. pachnodae xyl11A               YSFWTDAPGTVSMNL GSGGNYSTSWS--NT GNFVAGKGWST---- ---GSARTISYSGTF NPSGNA--YLAVYGW SHD-PLVEYYIVDSW  135
A. oryzae XylG1                   YSFWTNGGGDVEYTN GNGGQYSVKWT--NC DNFVAGKGWNP---- ---GSAKTVTYSGEW ESNSNS--YVSLYGW TQN-PLVEYYIVDKY  124
C. purpurea xyl1                  SSYWADYG-NTRYSC GAGGHYDLSWG--NG GNVVAGRGWKP---- ---ASPRAVTYSGSW QCNGNC--YLSVYGW TIN-PLVEYYIVENY  119
C. mixtus xyl                     YTFWKDSG-DASMGL QAGGRYTSQWSN-GT NNWVGGKGWNP---- --GG-PKVVTYSGSY NVDNSQNSYLALYGW TRS-PLIEYYVIESY  122
P. fluorescens cellulosa xyl      YTFWKDSG-DASMTL LSGGRYQSSWGN-ST NNWVGGKGWNP---- --GNNSRVISYSGSY GVDSSQNSYLALYGW TRS-PLIEYYVIESY  123
P. cochleariae xyl                YAFWKDSG-SATFTL ESGGRYAGNWTT-ST NNWVGGKGWNP---- --GNSWRTVNYSGYY GINEYANSYLSLYGW TTN-PLIEYYVVESY  114
A. kawachi xylC                   YVQNYNGNLGDFTYD ESAGTFSMYWEDGVS SDFVVGLGWTT---- --GS-SNAITYSAEY SASGSS-SYLAVYGW VNY-PQAEYYIVEDY   85
A. niger xyl1                     YVQNYNGNLGDFTYD ESAGTFSMYWEDGVS SDFVVGLGWTT---- --GS-SKAITYSAEY SASGSS-SYLAVYGW VNY-PQAEYYIVEDY  113
A. tubigensis xyl1                YVQNYNGNLGDFTYD ESAGTFSMYWEDGVS SDFVVGLGWTT---- --G--SSTITYSAEY SASGSA-SYLAVYGW VNY-PQAEYYIVEDY  112
P. purpurogenum xylB              YVQNYNGNLGAFSYN EGAGTFSMYWQQGVS NDFVVGLGRST---- --GS-SNPITYSASY SASGG--SYLAVYGW VNS-PQAEYYVVEAY  108
Cryptococcus sp S-2 xyl-CS2       YVQNYNGNVANFEYS QYDGTFSVNWNG--N TDFVCGLGWTV---- --GT-GRTITYSGSY NPGYSG-SYQAIYGW TGQGSLSEYYVIDNY  108
T. reesei xyl2                    YDQNYQT-GGQVSYS PSNTGFSVNWNT--Q DDFVVGVGWTT---- --GS-SAPINFGGSF SVNSGT-GLLSVYGW STN-PLVEYYIMEDN  133
B. pumilus xylA(1)                FELWKDYG-NTSMTL NNGGAFSASWNNIGN ALFRKGKKFDSTKTH HQ-LGNISINYNAAF NPGGNS--YLCVYGW TQS-PLAEYYIVESW  127
B. pumilus xylA(2)                YELWKDYG-NTSMTL NNGGAFSAGWNNIGN ALFRKGKKFDSTRTH HQ-LGNISINYNASF NPGGNS--YLCVYGW TQS-PLAEYYIVDSW  127
C. acetobutylicum XylB            YELWKDYG-NTSMTL KNGGAFSCQWSNIGN ALFRKGKKFNDTQTY KQ-LGNISVNYDCNY QPYGNS--YLCVYGW TSS-PLVEYYIVDSW  159
C. thermocellum XylB              FEYWKDTG-NGTMVL KDGGAFSCEWSNINN ILFRKGFKYDETKTH DQ-LGYITVTYSCNY QPNGNS--YLGVYGW TSN-PLVEYYIIESW  129
Bacillus sp 41M-1 xylJ            YEFWKDSGGSGSMTL NSGGTFSAQWSNVNN ILFRKGKKFDETQTH QQ-IGNMSINYGATY NPNGNS--YLTVYGW TVD-PLVEFYIVDSW  127
P. multivesiculatum xylA          YELWKDTG-TTSMTL LGGGKFSCSWSNINN CLFRIGKKWNCQYEW WE-LGTVLVNYDVDY NPNGNS--YLCIYGW TRN-PLVEYYIVESW  116
P. multivesiculatum xyl           ------------MTL LGGGKFSCNWSNIGN ALFRIGKKWDCTKTW QQ-LGTISVAYNVDY RPNGNS--YMCVYGW TRS-PLIEYYIVDSW   74
R. albus xylA                     YELWKDKG-DTEMTI NEGGTFSCKWSNINN ALFRRGKKFDCTKTY KE-LGNISVKYGVDY QPDGNS--YMCVYGW TID-PLVEFYIVESW  154
Caldicellulosiruptor sp xylD      YELWKDTG-NTTMTV DTGGRFSCQWSNINN ALFRTGKKFS--TAW NQ-LGTVKITYSATY NPNGNS--YLCIYGW SRN-PLVEFYIVESW  112
D. thermophilum xynB              YELWKDTG-NTTMTV YTQGRFSCQWSNINN ALFRTGKKYN--QNW QS-LGTIRITYSATY NPNGNS--YLCIYGW STN-PLVEFYIVESW  125
R. flavefaciens xylA              YEMWNQNGQGQASMN PGAGSFTCSWSNIEN FLARMGKNYDSQKKN YKAFGNIVLTYDVEY TPRGNS--YMCVYGW TRN-PLMEYYIVEGW  129
P. stipitis xylA                  YDRWTDLVGELEVRE LKHVMSHRTYSLCDL SCSTVLDSNS----- ---MFSLGKGWQAIS SRQGVG---ATVYGW TRSPLLIEYYVVDSW  151
```


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FIG. 2CONT'D

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                                  181          195 196          210 211          225 226          240 241          255 256          270
B. subtilis xylA                  GTYRPTGTYKG---- TVKSDGGTYDIYTTT RYNAPSIDGDRTTFT QYWSVRQSKRPTG-- --------SNATITF SNHVNAWKSHGMNLG    189
B. circulans xylA                 GTYRPTGTYKG---- TVKSDGGTYDIYTTT RYNAPSIDGDRTTFT QYWSVRQSKRPTG-- --------SNATITF TNHVNAWKSHGMNLG    189
B. stearothermophilus xylA        GTYRATGNYESG--- TVNSDGGTYDIYTTM RYNAPSIDG-TQTFQ QFWSVRQSKRPTG-- --------SNVSITF SNHVNAWRSKGMNLG    187
A. caviae xylA                    GTYRPTGTYKG---- TVNSDGGTYDIYTTM RYNAPSIDG-TQTFP QYWSVRQSKRPTG-- --------VNSTITF SNHVNAWPSKGMYLG    187
C. carbonum xyl1                  GTYDPSSQS--QNKG TVTSDGSSYKIAQST RTNQPSIDG-TRTFQ QYWSVRQNKRSSG-- -----------SVNM KTHFDAWASKGMNLG    196
H. turcicum xyl1                  GTYDPSSQA--QNKG TVTSDGSSYKIAQST RTNQPSIDG-TRTFQ QYWSVRQNKRSSG-- -----------SVNM KTHFDAWASKGMNLG    202
A. pisi xyl                       GTYDPSSQA--TVKG SVTADGSSYKIAQTQ RTNQPSIDG-TQTFQ QYWSVRQNKRSSG-- -----------SVNM KTHFDAWAAKGMKLG    202
S. commune xylA                   GSYDPSSAA--SHKG SVTCNGATYDILSTW RYNAPSIDG-TQTFE QFWSVRNPKKAPGG- S-------ISGTVDV QCHFDAWKGLGMNLG    175
T. lanuginosus xylA               GTYDPSSGA--TDLG TVECDGSIYRLGKTT RVNAPSIDG-TQTFD QYWSVRQDKRTSG-- -----------TVQT GCHFDAWARAGLNVN    198
C. carbonum xyl2                  GTYNPSSGA--QIKG SFQTDGGTYNVAVST RYNQPSIDG-TRTFQ QYWSVRTQKRVGG-- -----------SVNM QNHFNAWSRYGLNLG    206
C. sativus xyl2                   GTYNPSSGA--QVKG SFQTDGGTYNVAVST RYNQPSIDG-TRTFQ QYWSVRQQKRVGG-- -----------SVNM QNHFNAWSRYGLNLG    206
H. insolens xyl1                  GTYNPGSQA--QYKG TFYTDGDQYDIFVST RYNQPSIDG-TRTFQ QYWSIRKNKRVGG-- -----------SVNM QNHFNAWQQHGMPLG    202
M. grisea xyl22                   GEYNPGNSA--QSRG TLQAAGGTYTLHEST RVNQPSIEG-TRTFQ QYWAIRQQKRNSG-- -----------TVNT GEFFQAWERAGMRMG    207
C. gracile cgxA                   GTYDPSSQA--SKFG TIQQDGSTYTIAKTT RVNQPSIEG-TSTFD QFWSVRQNHRSSG-- -----------SVNV AAHFNAWAQAGLKLG    196
T. reesei xyl2(2)                 GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- -----------SVNT ANHFNAWAQQGLTLG    200
T. reesei, ALKO2721 Xyl2          GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- -----------SVNT ANHFNAWAQQGLTLG    200
T. reesei xyl1                    GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- -----------SVNT ANHFNAWAQQGLTLG    199
T. harzianum xylD                 GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- -----------SVNT ANHFNAWASHGLTLG    167
T. viride xyl                     GTYNPSTGT--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHAPAA-- -----------RSRL RTTSNAWRNLGLTLG    200
C. gracile cgxB                   GTYNPSTGA--TRLG SVTTDGSCYDIYRTQ RVNQPSIEG-TSTFY QFWSVRQNKRSGG-- -----------SVNM AAHFNAWAAAGLQLG    197
A. niger xyl2                     GDYNPGSGG--TYKG TVTSDGSVYDIYTAT RTNAASIQG-TATFT QYWSVRQNKRVG--- ----------GTVTT SNHFNAWAKLGMNLG    202
Penicillium sp 40 xylA            GDYNPGNSM--TYKG TVTSDGSVYDIYEHQ QVNQPSISG-TATFN QYWSIRQNTRSS--- ----------GTVTT ANHFNAWAKLGMNLG    198
Streptomyces sp xyl               GSYRPTG----EYRG TVYSDGGTYDIYKTT RYNAPSVEG-TRTFD QYWSVRQSKVIGS-- ----------GTITT GNHFDAWARAGMNLG    215
Streptomyces sp xyl1              GTYRPTG----TYKG TVTSDGGTYDVYQTT RVNAPSVEG-TKTFN QYWSVRQSKRTG--- ----------GSITA GNHFDAWARYGMPLG    204
S. thermocyaneoviolaceus xylB     GTYRPTG----TYKG TVYSDGGTYDIYMTT RYNAPSIEG-TKTFN QYWSVRQNKRTG--- ----------GTITT GNHFDAWAAHGMPLG    205
S. viridosporus T7A svxA          GTYRPTG----KYKG TVTSDGGTYDIYKTT RYNAPSIEG-TKTFD QYWSVRQSKRTG--- ----------GTITS GNHFDAWARNGMNLG    208
T. fusca xyl                      GTYRPTG----TYMG TVTTDGGTYDIYKTT RYNAPSIEG-TRTFD QYWSVRQSKRTS--- ----------GTITA GNHFDAWARHGMHLG    206
C. pachnodae xyl11A               GTYRPTG----TFMG TVNSDGGTYDIYKTT RTNAPSIEG-TATFT QYWSVRQSKRVG--- ----------GTITT ANHFNAWASHGMNLG    207
A. oryzae XylG1                   GDYDPSTGA--TELG TVESDGGTYKIYKTT RENAPSIEG-TSTFN QYWSVRQSGRVG--- ----------GTITA QNHFDAWANVGLQLG    198
C. purpurea xyl1                  GNYNPSAGA--QRRG QVTADGSIYDIYIST QHNQPSILG-TNTFH QYWSIRRNKRVGG-- -----------TVST GVHFNAWRSLGMPLG    193
C. mixtus xyl                     GSYNPASCSGGTDYG SFQSDGATYNVRRCQ RVQQPSIDG-TQTFY QYFSVRSPKKGFG-- Q-------ISGTITT ANHFNFWASKGLNLG    202
P. fluorescens cellulosa xyl      GSYNPASCSGGTDYG SFQSDGATYNVRRCQ RVNQPSIDG-TQTFY QYFSVRNPKKGFG-- N-------ISGTITF ANHVNFWASKGLNLG    203
P. cochleariae xyl                GSYSPLNCPGGTDEG SFTSGGATYQVRKCR RTNAPSIIG-TQSFD QYFSVRTPKKGFG-- Q-------VSGSVNF ADHVQYWASKGLPLG    194
A. kawachi xylC                   GDYNPCSS--ATSLG TVYSDGSTYQVCTDT RTNEPSITG-TSTFT QYFSVRESTRTSG-- -----------TVTV ANHFNFWAQHGFGNS    159
A. niger xyl1                     GDYNPCSS--ATSLG TVYSDGSTYQVCTDT RTNEPSITG-TSTFT QYFSVRESTRTSG-- -----------TVTV ANHFNFWAQHGFGNS    187
A. tubigensis xyl1                GDYNPCSS--ATSLG TVYSDGSTYQVCTDT RTNEPSITG-TSTFT QYFSVRESTRTSG-- -----------TVTV ANHFNFWAHHGFGNS    186
P. purpurogenum xylB              GNYNPCSSGSATNLG TVSSDGGTYQVCTDT RVNQPSITG-TSTFT QFFSVRQGSRTSG-- -----------TVTI ANHFNFWANDGFGNS    184
Cryptococcus sp S-2 xyl-CS2       GGYNPCTGSGVTQLG SLYSDGSSYQVCTHT QYNQPSIVG-TTTFP QYFSVRQNKRSSG-- -----------SVNM QNHFNYWAQHGFPNR    184
T. reesei xyl2                    -HNYPAQG---TVKG TVTSDGATYTIWENT RVNEPSIQG-TATFN QYISVRNSPRTSG-- -----------TVTV QNHFNAWASLGLHLG    205
B. pumilus xylA(1)                GTYRPTG----TYKG SFYADGGTYDIYETL RVNQPSIIG-DATFK QYWSVRQTKRTS--- ----------GTASV SEHFKKWESLGMPMG    199
B. pumilus xylA(2)                GTYRPTG----AYKG SFYADGGTYDIYETT RVNQPSIIG-IATFK QYWSVRQTKRTS--- ----------GTVSV SAHFRKWESLGMPMG    199
C. acetobutylicum XylB            GSWRPPGG---TSKG TITVDGGIYDIYETT RINQPSIQG-NTTFK QYWSVRRTKRTS--- ----------GTISV SKHFAAWESKGMPLG    232
C. thermocellum XylB              GTWRPPGA---TPKG TITVDGGTYEIYETT RVNQPSIKG-TATFQ QYWSVRTSKRTS--- ----------GTISV TEHFKAWERLGMKMG    202
Bacillus sp 41M-1 xylJ            GTWRPPGG---TPKG TINVDGGTYQIYETT RYNQPSIKG-TATFQ QYWSVRTSKRTS--- ----------GTISV SEHFRAWESLGMNMG    200
P. multivesiculatum xylA          GSWRPPGGS--P-MN TMYVDDGQYDVYVTD RINQPSIDG-NTNFK QYWSVRTQKKT---- ---------RGTVHV NHHFYNWQEMGLKVG    189
P. multivesiculatum xyl           GSWRPPGSN--S-MG TINVDGGTYDIYVTD RINQPSIDG-TTTFK QFWSVRTQKKT---- ---------SGVISV SKHFEAWTSKGLNLG    147
R. albus xylA                     GSWRPPGAA--ESLG TVTVDGGTYDIYKTT RYEQPSIDG-TKTFD QYWSVRQDKPTGDGT K-------IEGTISI SKHFDAWEQVGLTLG    234
Caldicellulosiruptor sp xylD      GSWRPPG-A--TSLG TVTIDGATYDIYKTT RVNQPSIEG-TRTFD QYWSVRTSKRTS--- ----------GTVTV TDHFKAWAAKGLNLG    185
D. thermophilum xynB              GNWRPPG-A--TSLG QVTIDGGTYDIYRTT RVNQPSIVG-TATFD QYWSVRTSKRTS--- ----------GTVTV TDHFRAWANRGLNLG    198
R. flavefaciens xylA              GDWRPPGND-GEVKG TVSANGNTYDIRKTM RYNQPSLDG-TATFP QYWSVRQTSGSANNQ TN-----YMKGTIDV TKHFDAWSAAGLDMS    212
P. stipitis xylA                  GSYHPSNTIT-GTFV TVKCDGGTYDIYTAV RVNAPSIEG--TTFT QYWSVRQSATIQLAV IKPLTLQNATITFTF SNHFDAWKTMTLEAT    238
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FIG. 2CONT'D

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                               271         285 286         300 301         315 316         330 331         345 346         360
B. subtilis xylA               SNWAYQVMATEGYQS SGSSNVTVW------ --------------- --------------- --------------- ---------------  213
B. circulans xylA              SNWAYQVMATEGYQS SGSSNVTVW------ --------------- --------------- --------------- ---------------  213
B. stearothermophilus xylA     SSWAYQVLATEGYQS SGRSNVTVW------ --------------- --------------- --------------- ---------------  211
A. caviae xylA                 NSWSYQVMATEGYQS SGNANVTVW------ --------------- --------------- --------------- ---------------  211
C. carbonum xyl1               -QHYYQIVATEGYFS TGNAQITVNCP---- --------------- --------------- --------------- ---------------  221
H. turcicum xyl1               -SHYYQIVATEGYFS SGSASITVNCP---- --------------- --------------- --------------- ---------------  227
A. pisi xyl                    -THNYQIVATEGYFS SGSAQITVNCA---- --------------- --------------- --------------- ---------------  227
S. commune xylA                SEHNYQIVATEGYQS SGTATITVTAS---- --------------- --------------- --------------- ---------------  201
T. lanuginosus xylA            GDHYYQIVATEGYFS SGYARITVADVG--- --------------- --------------- --------------- ---------------  225
C. carbonum xyl2               -QHYYQIVATEGYQS SGSSDIYVQTQ---- --------------- --------------- --------------- ---------------  231
C. sativus xyl2                -QHYYQIVATEGYQS SGSSDIYVQTQ---- --------------- --------------- --------------- ---------------  231
H. insolens xyl1               -QHYYQVVATEGYQS SGESDIYVQTH---- --------------- --------------- --------------- ---------------  227
M. grisea xyl22                -NHNYMIVATEGYRS AGNSNINVQTPA--- --------------- --------------- --------------- ---------------  233
C. gracile cgxA                -SHNYQIVATEGYQS SGSSSITVS------ --------------- --------------- --------------- ---------------  219
T. reesei xyl2(2)              -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- ---------------  223
T. reesei, ALKO2721 Xyl2       -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- ---------------  223
T. reesei xyl1                 -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- ---------------  222
T. harzianum xylD              -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- ---------------  190
T. viride xyl                  -TLDYQIIAVEGYFS SGNANINVS------ --------------- --------------- --------------- ---------------  223
C. gracile cgxB                -THDYQIVATEGYYS SGSATVNVGASSDGS TGGGSTGGGSTNVSF --------------- --------------- ---------------  241
A. niger xyl2                  THN-YQIVATEGYQS SGSSSITVQ------ --------------- --------------- --------------- ---------------  225
Penicillium sp 40 xylA         SFN-YQIVSTEGYES SGSSTITVS------ --------------- --------------- --------------- ---------------  221
Streptomyces sp xyl            QFQYYMIMATEGYQS SGSSNITVSG----- --------------- --------------- --------------- ---------------  240
Streptomyces sp xyl1           SFNYYMIMATEGYQS SGSSSISVS------ --------------- --------------- --------------- ---------------  228
S. thermocyaneoviolaceus xylB  TFN-YMILATEGYQS SGSSNITVGDSGGDN GGGGG---------- --------------- --------------- ---------------  292
S. viridosporus T7A svxA       NHN-YMIMATEGYQS SGSSTITVSESGSGG GGGGG---------- --------------- --------------- ---------------  286
T. fusca xyl                   THD-YMIMATEGYQS SGSSNVTLGTSGGGN PGGGN---------- --------------- --------------- ---------------  295
C. pachnodae xyl11A            RHD-YQILATEGYQS SGSSNITIGSTSGGG GSGGG---------- --------------- --------------- ---------------  293
A. oryzae XylG1                THN-YMILATEGYKS SGSATITVE------ --------------- --------------- --------------- ---------------  221
C. purpurea xyl1               -TYDYMIVATEGFRS SGSASITVS------ --------------- --------------- --------------- ---------------  216
C. mixtus xyl                  -NHDYMVLATEGYQS RGSSDITVSEGTGGT TSSSV---------- --------------- --------------- ---------------  290
P. fluorescens cellulosa xyl   -NHNYQVLATEGYQS RGSSDITVSEGTSGG GTSSV---------- --------------- --------------- ---------------  292
P. cochleariae xyl             -THAHQIFATEGYQS SGFADITVS------ --------------- --------------- --------------- ---------------  217
A. kawachi xylC                DFN-YQVMAVEAWSG AGSASVTIS------ --------------- --------------- --------------- ---------------  182
A. niger xyl1                  DFN-YQVMAVEAWSG AGSASVTISS----- --------------- --------------- --------------- ---------------  211
A. tubigensis xyl1             DFN-YQVVAVEAWSG AGSASVTISS----- --------------- --------------- --------------- ---------------  210
P. purpurogenum xylB           NFN-YQVVAVEAWSG TGTASVTVSA----- --------------- --------------- --------------- ---------------  208
Cryptococcus sp S-2 xyl-CS2    NFN-YQVLAVEGFSG SGNANMKLISG---- --------------- --------------- --------------- ---------------  209
T. reesei xyl2                 QMN-YQVVAVEGWGG SGSASQSVSN----- --------------- --------------- --------------- ---------------  229
B. pumilus xylA(1)             -KMYETALTVEGYRS NGSANVMTNQLMIR- --------------- --------------- --------------- ---------------  227
B. pumilus xylA(2)             -KMYETAFTVEGYQS SGSANVMTNQLFIGN --------------- --------------- --------------- ---------------  228
C. acetobutylicum XylB         -KMHETAFNIEGYQS SGKADVNSMSINIGK --------------- --------------- --------------- ---------------  261
C. thermocellum XylB           -KMYEVALVVEGYQS SGKADVTSMTITVGN APS------------ --------------- --------------- ---------------  277
Bacillus sp 41M-1 xylJ         -NMYEVALTVEGYQS SGSANVYSNTLTIGG Q-------------- --------------- --------------- ---------------  268
P. multivesiculatum xylA       -KVYEASLNIEGYQS AGSATVNKNEVVQT- --------------- --------------- --------------- ---------------  217
P. multivesiculatum xyl        -LMYEASLTIEGYQS SGSATVNQNDVTGG- --------------- --------------- --------------- ---------------  175
R. albus xylA                  -NMYEVALNIEGYQS NGQATIYENELTVDG NY------------- --------------- --------------- ---------------  303
Caldicellulosiruptor sp xylD   -TIDQITLCVEGYQS SGSANITQNTFTIGG SSSGSSNGSNNG--- --------------- --------------- ---------------  274
D. thermophilum xynB           -TIDQITLCVEGYQS SGSANITQNTFSQG- ----SSSGSS----- --------------- --------------- ---------------  273
R. flavefaciens xylA           GTLYEVSLNIEGYRS NGSANVKSVSVTQGG SSDNGGQQQNNDW-- --------------- --------------- ---------------  255
P. stipitis xylA               HSTEGYFSSGITYEQ PHQPHRNTWATSLTS QTKHTARSLPIN--- --------------- --------------- ---------------  328
```


NZAS-0206258

SEQUENCE LISTINGS

The amino acid sequence of the mature *Bacillus subtilis* xylanase (SEQ ID. No. 1):

```
5   1        10 11        20 21        30 31        40 41        50 51        60
    ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT INYNAGVWAP

    61       70 71        80 81        90 91       100 101      110 111      120
    NGNGYLTLYG WTRSPLIEYY VVDSWGTYRP TGTYKGTVKS DGGTYDIYTT TRYNAPSIDG
10
    121     130 131      140 141      150 151      160 161      170 171      180
    DRTTFTQYWS VRQSKRPTGS NATITFSNHV NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS

    181
15  NVTVW
```

Amino acid sequences derived from wheat flour xylanase inhibitor

A chain of inhibitor

20

N-terminal:

GAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNV (35aa) SEQ ID NO. 2

C-terminal:

25 KRLGFSRLPHFTGCGGL (17aa) SEQ ID NO. 3

B chain of inhibitor

N-terminal:

30 LPVPAPVTKDPATSLYTIPFH (21aa) SEQ ID NO. 4

Lys-C digested B Chain:

LLASLPRGSTGVAGLANSGLALPAQVASAQK (31aa) SEQ ID NO. 5

35 GGSPAHYISARFIEVGDTRVPSVE (24aa) SEQ ID NO. 6

VNVGVLAACAPSK (13aa) SEQ ID NO. 7

VANRFLLCLPTGGPGVAIFGGGPVPWPQFTQSMPYTLVVVK SEQ ID NO. 8

Thermomyces lanuginosus xylanase A gene (SEQ ID NO. 9)

```
1        10 11        20 21        30 31        40 41        50 51        60
ATGCAGACAA CCCCCAACTC GGAGGGCTGG CACGATGGTT ATTACTATTC CTGGTGGAGT

61       70 71        80 81        90 91       100 101      110 111      120
GACGGTGGAG CGCAGGCCAC GTACACCAAC CTGGAAGGCG GCACCTACGA GATCAGCTGG

121     130 131      140 141      150 151      160 161      170 171      180
GGAGATGGCG GTAACCTCGT CGGTGGAAAG GGCTGGAACC CCGGCCTGAA CGCAAGAGCC

181     190 191      200 201      210 211      220 221      230 231      240
ATCCACTTTG AGGGTGTTTA CCAGCCAAAC GGCAACAGCT ACCTTGCGGT CTACGGTTGG

241     250 251      260 261      270 271      280 281      290 291      300
ACCCGCAACC CGCTGGTCGA GTATTACATC GTCGAGAACT TTGGCACCTA TGATCCTTCC

301     310 311      320 321      330 331      340 341      350 351      360
TCCGGTGCTA CCGATCTAGG AACTGTCGAG TGCGACGGTA GCATCTATCG ACTCGGCAAG

361     370 371      380 381      390 391      400 401      410 411      420
ACCACTCGCG TCAACGCACC TAGCATCGAC GGCACCCAAA CCTTCGACCA ATACTGGTCG

421     430 431      440 441      450 451      460 461      470 471      480
GTCCGCCAGG ACAAGCGCAC CAGCGGTACC GTCCAGACGG GCTGCCACTT CGACGCCTGG

481     490 491      500 501      510 511      520 521      530 531      540
GCTCGCGCTG GTTTGAATGT CAACGGTGAC CACTACTACC AGATCGTTGC AACGGAGGGC

541     550 551      560 561      570 571      580 581  588
TACTTCAGCA GCGGCTATGC TCGCATCACC GTTGCTGACG TGGGCTAA
```

NZAS-0206260

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 01/00426

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 00 39289 A (SOERENSEN JENS FRISBAEK ;DANISCO (DK); SIBBESEN OLE (DK)) 6 July 2000 (2000-07-06) example 2 seq no 7, 9, 11 ---	1-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2001	24 08. 2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2, NL - 2280 HV Rijswijk, Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Carolina Palmcrantz

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Form PCT/ISA/210 (second sheet) (July 1992)

NZAS-0206261

INTERNATIONAL SEARCH REPORT

Int Application No
PCT/IB 01/00426

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	DATABASE STN INTERNATIONAL [Online] MCLAUCHLAN W R ET AL: "Xylanase inhibitors from cereals: implications for baking, brewing and plant technology" retrieved from CAPLUS, accession no. 2001:287270 XP002901834 abstract -& MCLAUCHLAN W R ET AL: "Xylanase inhibitors from cereals: implication for baking, brewing and plant technology" VTT SYMP (2000) 207 2ND EUROPEAN SYMPOSIUM ON ENZYMES IN GRAIN PROCESSING, 8 - 10 December 1999, pages 55-61, XP002901835 HELSINKI / FINLAND ISSN: 0357-9387 page 58 "current and future studies" pages 59,60 "summary" ---	1-22
A	MCLAUCHLAN W R ET AL: "A novel class of protein from wheat which inhibits xylanases" BIOCHEM J., vol. 338, - 1999 pages 441-446, XP002901836 introduction ---	1-22
A	EP 0 979 830 A (TNO) 16 February 2000 (2000-02-16) page 3, line 3 - line 5; claim 7 -----	1-22

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

NZAS-0206262

INTERNATIONAL SEARCH REPORT

ational application No.
PCT/IB 01/00426

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [ ] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. [X] Claims Nos.: 1-22
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-22

Present claims 1-22 relate to a very large number of variant xylanase polypeptides since there are many known xylanase inhibitors and the xylanase may be modified in a number of different ways to the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is not to be found for any specific variant xylanase polypeptide. In the present case, the claims so lack support, and the application so lacks diclosure, that a meaningful search over the whole of the claimed scope is impossible.
Consequently, the search has been focused on the general idea underlying the invention.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

ɔrmation on patent family members

Int nal Application No
PCT/IB 01/00426

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0039289 A	06-07-2000	AU 1676600 A	31-07-2000
		FR 2788781 A	28-07-2000
EP 0979830 A	16-02-2000	NONE	

Form PCT/SA/210 (patent family annex) (July 1992)

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